UNIVERSITÀ DEGLI STUDI DI PALERMO

DIPARTIMENTO DI SCIENZE AGRARIE E FORESTALI

Dottorato di Ricerca in Frutticoltura Mediterranea

ESSENTIAL OILS AS MULTI-TARGET COMPOUNDS FOR NOVEL FOOD SAFETY STRATEGIES

SSD AGR/16 – Microbiologia Agraria

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CICLO XXVI - 2015

DOTTORATO

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Introduction

Introduction

1. Foodborne outbreaks and food safety

Access to sufficient and safe food is a basic human necessity. Serious outbreaks of foodborne disease have been documented on every continent in the past decade, including Europe (Figure 1), illustrating the public health and social significance of these diseases. Foodborne diseases not only adversely affect people's health and well-being, but also have negative economic consequences for individuals, families, communities, businesses and countries. It is noteworthy to report that foodborne and waterborne diarrheal diseases kill an estimated 2.2 million people annually, most of whom are children (WHO, 2014). Diarrhoea is the most common foodborne illness caused by foodborne pathogens, but other serious consequences include kidney and liver failure, brain and neural disorders, reactive arthritis and death.

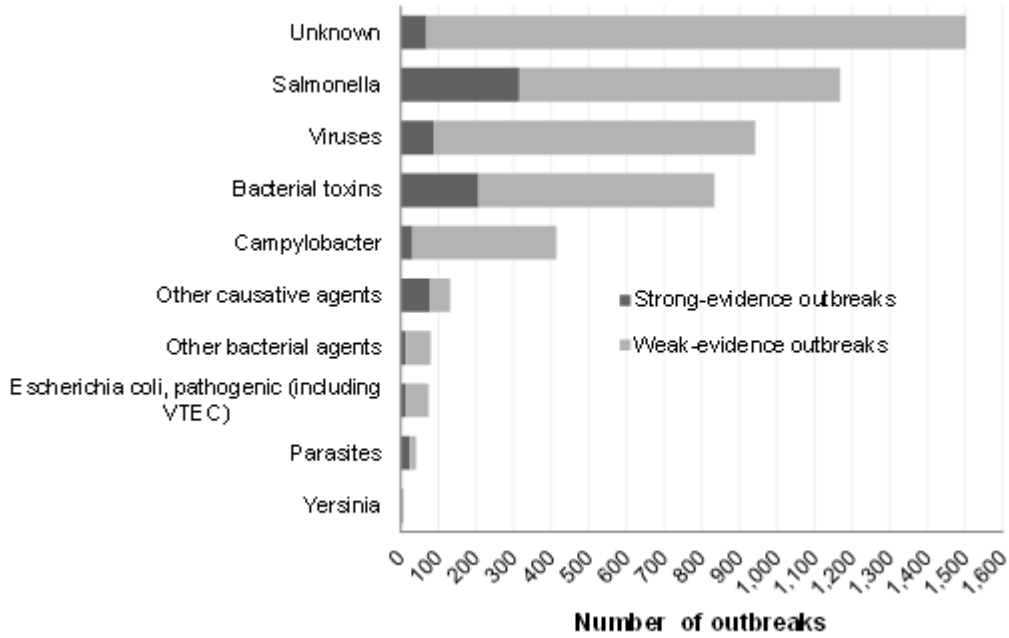


Figure 1. Distribution of foodborne outbreaks per causative agents in the EU (EFSA Journal, 2015).

The full spectrum of the burden of foodborne diseases has been never quantified on a global basis, since foodborne illnesses are often under-reported. Traditionally, the term "foodborne disease" has been used for illnesses caused by microorganisms, with often acute reactions.

Nowadays, the term foodborne disease is often used in a wide, all-encompassing sense including different causative agents (such as bacteria, viral, fungal or parasitic nature) and as well, other risks associated with food along the entire food chain (as chemical or prionic, "PRoteinaceus Infective ONly" particle, contaminations).

The past decade has seen new challenges to food safety. The integration and consolidation of agricultural and food industries, new dietary habits, the globalization of the food trade and human movements are modifying the patterns of food production, distribution and consumption.

The globalization of the food trade offers many benefits to consumers, as it can bring to the market a wider variety of foods that are accessible, affordable and meet consumer demands. At the same time, these changes present new challenges to food safety and have widespread repercussions for health, for instance by creating an environment in which both known and new or emerging foodborne diseases may occur in greater magnitude. Other key challenges include increasing awareness of the health impact of antimicrobial resistance in foodborne pathogens; identifying and assessing the risks posed by newly identified pathogenic microorganisms in the food supply (WHO, 2014).

Considering the increasing interest of the consumer towards natural products, essential oils may constitute effective alternatives or complements to synthetic

compounds, without showing resistance effects (Carson *et al.*, 2001; Nostro *et al.*, 2004; Mulyaningsih *et al.*, 2010). In this context, the hurdle technology, a conservation strategy for food based on the combination of different preservation techniques (Rico *et al.*, 2007), seems to fulfil both consumer and industrial needs.

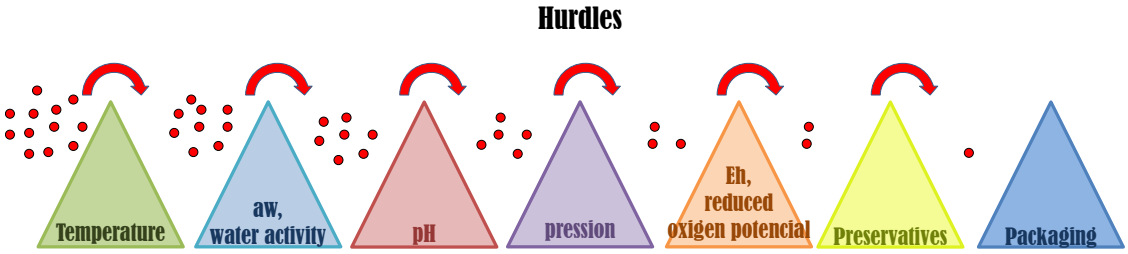


Figure 2. Common hurdles used in food preservation technologies (adapted from Leistner, 1999).

The control of temperature, water activity, acidity, redox potential and the use of preservatives, modified atmosphere and competitive microorganisms (e.g., lactic acid bacteria) represent the most important hurdles commonly used for food preservation (Figure 2; Leistner, 1999).

By using hurdles, the intensity of a certain preservation technique can be kept relatively low, minimizing the loss of quality, while the overall impact on microbial growth may remain the same or be better (Rico *et al.*, 2007). The most important factor to consider is the selection of hurdles; this choice should be done carefully on the basis of the quality attributes of a product (Gorris *et al.*, 1999). According to Leistner (1999), there are more than 60 potential hurdles for foods that improve the stability and/or quality of minimally processed products.

Food safety and food control systems need to adapt to today's food production and distribution practices, moving their focus gradually from the end-product to the

process control throughout the food chain. Therefore, food safety must be systematically integrated into policies and interventions to improve nutrition and food security.

2. Essential oils

Essential oils (EOs) have been known since antiquity for their flavour properties and, therefore, used mainly as perfumes. EOs were extracted by distillation since more than 2000 years ago in Egypt, India and Persia and thereafter the Arabs improved the extraction method (Guenther, 1948). Undeniably, spices and related EOs have been also used for preservative and medical purposes since ancient times, but their trade began only in the 13th century and they emerged widespread in Europe in the 16th sold in pharmacies as medical preparations (Crosthwaite, 1998). Even though the bactericidal properties of EOs have been investigated since 1881 by De la Croix (Boyle, 1955), in the recent past their use was still as aroma and flavoring compounds principally (Guenther, 1948).

Nowadays, in the European Union, EOs are mainly used in food as flavourings, in perfumes and in pharmaceuticals for their functional properties (Bauer *et al.*, 2001; Van de Braak *et al.*, 1999; Van Welie, 1997). As well, they are used in aromatherapy due to its psycho-emotional effect and they constitute approximately 2% of the total market (Van de Braak *et al.*, 1999).

Scientifically, EOs, also called volatile or ethereal oils, are defined as aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Guenther, 1948). They are produced by plants as secondary

metabolites and often accumulated in a glycosidic form in vacuoles or in secretory structures (Figueiredo *et al.*, 2008). EOs play an important ecological role having antibacterials, antivirals, antifungals, insecticides properties and they also act against herbivores by reducing their appetite for such plant, resulting in a broad protection for the plant. For example, a recent research demonstrated that *Penicillium* infection on mandarin determines an increase in the EOs emission as volatile compounds, especially of haliphatic esters and alcohols, branched esters and α-farneseno, a linear sesquiterpen (Gurrea Martínez, 2014). Besides, they may also act as "favoring" compound, attracting insects to improve the dispersion of pollens and seeds (Palazzolo *et al.*, 2013). These ecological properties have been confirmed after the extraction of the EOs from the plant. Indeed, antibacterial (Carson *et al.*, 1995; Deans *et al.*, 1987; Mourey *et al.*, 2002), antiviral (Bishop, 1995; Elizaquível *et al.*, 2013), antitoxigenic (Akgül *et al.*, 1991; Juglal *et al.*, 2002; Ultee *et al.*, 2001), antiparasitic (Pandey *et al.*, 2000; Pessoa *et al.*, 2002), and insecticidal (Karpouhtsis *et al.*, 1998; Konstantopoulou *et al.*, 1992) properties are possibly related to the function of these compounds in plants (Guenther, 1948; Mahmoud *et al.*, 2002).

Once extracted, they result in natural mixtures of lipophilic substances, containing about 20-60 components, whose only a few at high concentrations (20-70%) compared to others (Palazzolo *et al.*, 2013).

EOs can be extracted from plants using various methods as expression, fermentation, enfleurage, cold pressing or extraction (water, steam or organic solvent extraction) but the steam distillation is the most commonly used for commercial production of EOs (Van de Braak *et al.*, 1999). Nowadays, other methods are available such as

supercritical CO_2, by which EOs are extracted at lower temperature to avoid potential damage to desired compounds at high temperatures (Espinosa *et al.*, 2000; Gao *et al.*, 2005; Mira *et al.*, 1996). The method used to extract EOs greatly affects their chemical profile (number and stereo chemistry of extracted molecules). Hence, the choice of extraction method depends also on the purpose of the use (Palazzolo *et al.*, 2013). Therefore, EOs properties depend on their chemical composition (Lanciotti *et al.*, 2004; Moreira *et al.*, 2005; Espina *et al.*, 2011) which is influenced by the raw plant material and extraction method (Burt, 2004). Genotype, part of the plant, geographical, ecological conditions and cultural techniques are other factors affecting the chemical composition of EOs and their properties.

On a commercial point of view, the application of EOs fulfils consumers' demand for naturally processed foods, with fewer synthetic additives and with a smaller impact on the environment. This trend of 'green' consumerism is being experiencing in Western industrialized countries (Burt, 2004).

As a more serious health issue, there is a need for decreasing the foodborne health risks (WHO, 2014) by using new bactericides effective against food-related pathogens and as well the increasing number of resistant strains (Moreira *et al.*, 2005; Fisher *et al.*, 2008; Ponce *et al.*, 2011). Besides, the improvements in food production techniques and the global trade have revealed new foodborne health risks. It has been estimated that as many as 30% of people in industrialized countries suffer from a foodborne disease each year and in 2000 at least two million people died from diarrhoeal disease worldwide (WHO, 2002). Novel recommendations of the Codex Alimentarius provide guidance on the controls and associated tools that can be

adopted by regulators and industry to minimize the likelihood of illnesses arising from the consumption of ready-to-eat foods (CAC, 2007) and they converge on the reduction of the risk through safe food preparation, consumption and storage practices.

Today, only a few food preservatives containing EOs are already commercially available. To our knowledge, DOMCA S.A. (Alhendín, Granada, Spain; (Mendoza-Yepes *et al.*, 1997)) and Bavaria Corp. (Apopka, FL, USA; (Cutter, 2000)) result the only companies producing generally recognized as safe (GRAS) food additives containing EOs.

3. Composition of EOs

Numerous publications have presented data on the composition of the various EOs. Bauer *et al.* (2001) summarized the major components of the economically interesting EOs. Detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the EO or its headspace (Daferera *et al.*, 2000; Delaquis *et al.*, 2002). EOs can comprise more than sixty individual components (Russo *et al.*, 1998; Senatore, 1996). Major components can constitute up to 85 % of the EO whereas other components are present only in traces (Bauer *et al.*, 2001; Senatore, 1996). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Cosentino *et al.*, 1999). In Table 1 are reported the major components of *Citrus* EOs showing biological properties.

Furthermore, there is some evidence that minor components of EOs have a critical role to play in their antimicrobial activity, possibly by producing a synergistic effect

between other components. This has been found to be the case for *Citrus* (Settanni *et al.*, 2014), sage (Marino *et al.*, 2001), certain species of *Thymus* (Lattaoui *et al.*, 1994; Marino *et al.*, 1999; Paster *et al.*, 1995) and oregano (Paster *et al.*, 1995). Besides, the biological activity of an oil can be relate to the stereochemical configuration of its components, to the proportion in which they are present and to how they interact among them (Delaquis *et al.*, 2002; Dorman *et al.*, 2000; Marino *et al.*, 2001). Some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (Gill *et al.*, 2002; Mourey *et al.*, 2002), which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence.

Moreover, as mentioned above, the composition of EOs from a particular species of plant can differ between harvesting seasons (Settanni *et al.*, 2014) and between geographical sources (Arras *et al.*, 1992; Cosentino *et al.*, 1999; Faleiro *et al.*, 2002; Juliano *et al.*, 2000; Marino *et al.*, 1999; McGimpsey *et al.*, 1994). This can be explained, at least in part, by the formation of antimicrobial substances from their precursors. p-Cymene (1-methyl-4-(1-methylethyl)-benzene) and γ-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the precursors of carvacrol (2-methyl-5-(1-methylethyl) phenol) and thymol (5-methyl- 2-(1-methylethyl)phenol) in species of *Origanum* and *Thymus* (Cosentino *et al.*, 1999; Jerkovic *et al.*, 2001; Ultee *et al.*, 2002).

In *Citrus* Genus (*Rutaceae* family), the main EOs compounds are the monoterpenes and, among them, the limonene, a cyclic monoterpene, is the most abundant ranging up to 85% (Gurrea Martínez, 2014). On the other hand, the sesquiterpenes result to

show a large diversity among *Citrus* species. *Citrus* EOs are biosynthesized in spherical secretory cavities known as oil glands (Palazzolo *et al.*, 2013), mainly diffused in primary tissues of the shoot (*i.e.* leaf, thorns, prophyllis, sepals, etc.) (Schneider, 1968) and, particularly, in flavedo. Even though the *Citrus* species show different oil glands density, as well at cultivar level, the amount of EOs produced was not related to the number of these secretory structures (Germanà *et al.*, 1995), at least in lemon. Different results have been achieved comparing two mandarin clones, one of which (*deg*) was a mutant with decreased oil glands (Gurrea Martínez, 2014). In this case, significant quantitative differences were detected between clones regarding the amount of EOs produced, even if the released ones as volatile compounds did not.

Enantiomers of EO components have been shown to exhibit antimicrobial activity to different extents (Lis-Balchin *et al.*, 1999; Palazzolo *et al.*, 2013).

The EO components can be divided into two different classes based on biosynthetic origin (Croteau *et al.*, 2000; Betts, 2001; Pichersky *et al.*, 2006). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight (Bakkali *et al.*, 2008) The terpenes have different classes from a structural and functional point of view. They are substances composed of isoprene (2-methylbutadiene) units. Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups (Caballero *et al.*, 2003). They can be hydrocarbons, alcohols, aldehydes, ketones, acids, acetals, esters, lactones, epoxides, ethers or phenols; they can also contain sulfur and nitrogen groups, can be saturated

or unsaturated, with a linear, branched, cyclic or heterocyclic structure, and with a greater or lesser number of carbon atoms. In general, only the hemiterpenoids (5 carbon atoms), monoterpenoids (10 carbon atoms) and sesquiterpenoids (15 carbon atoms) are sufficiently volatile to be components of essential oils. Monoterpenoids are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures, for example carbures, alcohols, aldehydes, ketone, esters, ethers, peroxides, phenols (Bakkali *et al.*, 2008). When the molecule is optically active, the two enantiomers are very often present in different plants.

The aromatic compounds, compared to terpenes and terpenoids, are derivatives of phenylpropane, which are less frequently than the terpenes in essential oils. The biosynthetic pathways concerning terpenes and phenylpropanic derivatives are generally separated in plants but they may coexist in some, with one major pathway taking over (Bakkali *et al.*, 2008).

Table 1. Chemical compounds of *Citrus* EOs and their biological activities (adapted from Burt, 2004 and Jing *et al.*, 2014).

#	Name	Class	Plant source	Bioactivity	Reference
1	α-pinene	monoterpene	*Citrus* species; *Rosmarinus officinalis; Salvia officinalis*	antimicrobial antifungal	Hosni *et al.*, 2010; Lota *et al.*, 2002; Daferera *et al.*, 2000; Daferera *et al.*, 2003; Pintore *et al.*, 2002; Marino *et al.*, 2001.
2	α -thujene	monoterpene	*Citrus* species		Jabalpurwala *et al.*, 2009; Lota et al., 2002.
3	camphene	monoterpene	*Citrus* species		Lota *et al.*, 2002; Bourgou *et al.*, 2012; Sawamura *et al.*, 1991.
4	β-pinene	monoterpene	*Citrus* species; *Salvia officinalis*	antifungal antimicrobial	Jabalpurwala *et al.*, 2009; Lota *et al.*, 2002; Hammer *et al.*, 2003; Marino *et al.*, 2001.
5	sabinene	monoterpene	*Citrus* species	antifungal	Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Espinosa-García *et al.*, 1991.
6	α-phellandrene	monoterpene	*Citrus* species	insecticidal activity	Hosni *et al.*, 2010; Park *et al.*, 2003.
7	δ-3-carene	monoterpene	*Citrus* species	anti-inflammatory	Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Ocete *et al.*, 1989.
8	α-myrcene	monoterpene	*Citrus* species		Hosni *et al.*, 2010; Lota *et al.*, 2002.
9	β-myrcene	monoterpene	*Citrus* species	antifungal	Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Tao *et al.*, 2014.
10	α-terpinene	monoterpene	*Citrus* species		Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
11	γ-terpinene	monoterpene	*Citrus* species, *Eucaliptus* spp; *Origanum vulgare; Thymus vulgaris*	antimicrobial antifungal antiviral	Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Sartorelli *et al.*, 2007. Charai *et al.*, 1996; Daferera *et al.*, 2000; Marino *et al.*, 2001.
12	limonene	monoterpene	*Citrus* species	anti-inflammatory, antioxidant, antimicrobial	Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
13	1,8-cineole	monoterpenic oxide	*Citrus limon*; *Rosmarinus officinalis; Salvia officinalis*	anti-inflammatory	Lota *et al.*, 2002; Santos *et al.*, 2000; Daferera *et al.*, 2000; Marino *et al.*, 2001.
14	(Z)-ocimene	monoterpene	*Citrus* species		Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
15	(E)-ocimene	monoterpene	*Citrus* species		Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
16	trans-sabinene	monoterpene	*Citrus* species		Hosni *et al.*, 2010; Lota *et al.*, 2002; Vekiari *et al.*, 2002.
17	cis-sabinene	monoterpene	*Citrus* species		Hosni *et al.*, 2010.
18	p-cymene	monoterpene	*Citrus* species; *Origanum vulgare; Thymus vulgaris*	antimicrobial antiviral	Hosni *et al.*, 2010; Lota *et al.*, 2002; Vekiari *et al.*, 2002; Sartorelli *et al.*, 2007; Daferera *et al.*, 2000; Daferera *et al.*, 2003; Marino *et al.*, 2001.
19	α-terpinolene	monoterpene	*Citrus* species		Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
20	linalool	monoterpene alcohol	*Citrus* species; *Coriandrum sativum*	antimicrobial	Hosni *et al.*, 2010; Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Delaquis *et al.*, 2002.
21	trans-pinocarveol	monoterpenoid	*Citrus* species		Hosni *et al.*, 2010.
22	neo-allo-ocimene	monoterpene	*Citrus* species		Jabalpurwala *et al.*, 2009.
23	allo-ocimene	monoterpene	*Citrus* species		Jabalpurwala *et al.*, 2009.
24	(Z)-epoxy-ocimene		*Citrus* species		Jabalpurwala *et al.*, 2009.
25	terpinen-4-ol	monoterpenic oxide	*Citrus* species	anti-inflammatory	Lota *et al.*, 2002; Lahlou *et al.*, 2003.
26	β-cyclocitral	sesquiterpenoid	*Citrus* species	antimicrobial	Hosni *et al.*, 2010; Proszenyak *et al.*, 2007.
27	cis-linalool oxide	monoterpenic oxide	*Citrus* species		Lota *et al.*, 2002.

28	α-p-dimethylstyrene		*Citrus* species		Jabalpurwala *et al.*, 2009.
29	sabinene hydrate	monoterpene	*Citrus* species		Jabalpurwala *et al.*, 2009.
30	trans-para-menth-2-ene-1-ol	monoterpene alcohol	*Citrus* species		Hosni *et al.*, 2010.
31	carvacryl methyl oxide	monoterpene phenol derivate	*Citrus* species		Hosni *et al.*, 2010.
32	(Z)-limonene oxide	monoterpenic oxide	*Citrus* species		Jabalpurwala *et al.*, 2009.
33	δ-elemene	sesquiterpene hydrocarbon	*Citrus* species	anticancer	Jabalpurwala *et al.*, 2009.
34	β-elemene	sesquiterpene hydrocarbon	*Citrus* species		Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
35	α-copaene	sesquiterpene hydrocarbon	*Citrus* species	attractant for male fruit flies	Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009; Nishida *et al.*, 2000.
36	β-copaene	sesquiterpene hydrocarbon	*Citrus* species		Hosni *et al.*, 2010.
37	(E)-(E)-2,4-decadienal		*Citrus* species		Hosni *et al.*, 2010.
38	α-bergamotene	sesquiterpene hydrocarbon	*Citrus limon*		Jabalpurwala *et al.*, 2009.
39	trans carveol	monoterpenoid alcohol	*Citrus* species		Hosni *et al.*, 2010.
40	(E)-caryophyllene	sesquiterpene hydrocarbon	*Citrus* species	antimicrobial	Lota *et al.*, 2002; Juliani *et al.*, 2002.
41	β-caryophyllene	sesquiterpene hydrocarbon	*Citrus* species	antioxidant, anticancer, antibiotic, anti-inflammatory	Jabalpurwala *et al.*, 2009; Legault *et al.*, 2007.
42	trans α-bergamotene	sesquiterpene hydrocarbon	*Citrus* species		Hosni *et al.*, 2010.
43	β-ionone	isoprenoid	*Citrus* species	antimicrobial	Hosni *et al.*, 2010; Radulovic *et al.*, 2006.
44	β-farnesene	sesquiterpene	*Citrus* species		Jabalpurwala *et al.*, 2009.
45	(E)-β-farnesene	sesquiterpene	*Citrus limon*	kairomone for the ladybird	Lota *et al.*, 2002; Francis *et al.*, 2004.
46	(E,E)-α-farnesene	sesquiterpene	*Citrus limon*	attractant for lepidopteran	Lota *et al.*, 2002; Pechous *et al.*, 2004.
47	farnesol	sesquiterpene	*Citrus* species	anticancer	Jabalpurwala *et al.*, 2009; Burke *et al.*, 1997.
48	α-humulene	sesquiterpene	*Citrus* species	anticancer	Lota *et al.*, 2002; Hosni *et al.*, 2010; Legault et al., 2007.
49	β-bisabolene	sesquiterpene	*Citrus* species		Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
50	geranial	sesquiterpene aldeyde	*Citrus* species	antifungal	Lota *et al.*, 2002; Wuryatmo *et al.*, 2003; Jabalpurwala *et al.*, 2009.
51	geraniol	terpene alcohol	*Citrus* species	antimicrobial, antioxidant, anticancer, anti-inflammatory	Lota *et al.*, 2002; Chen *et al.*, 2010; Jabalpurwala *et al.*, 2009.
52	α-citronellol	monoterpene alcohol	*Citrus* species		Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
53	β-citronellol	monoterpene alcohol	*Citrus* species		Lota *et al.*, 2002; Jabalpurwala *et al.*, 2009.
54	curcumene	sesquiterpene hydrocarbon	*Citrus* species	anti-inflammatory	Lota *et al.*, 2002; Chavan *et al.*, 2010; Mujumdar *et al.*, 2004; Jabalpurwala *et al.*, 2009.
55	neral	monoterpene alcohol	*Citrus limon*	antifungal	Lota *et al.*, 2002; Wuryatmo *et al.*, 2003.
56	nerol	monoterpene aldehyde	*Citrus* species	antimicrobial	Lota *et al.*, 2002; Kotan *et al.*, 2007; Jabalpurwala *et al.*, 2009.
57	calamenene	sesquiterpene	*Citrus limon*	anticancer	Dai *et al.*, 2012; Jabalpurwala *et al.*, 2009.
58	(Z)-jasmone		*Citrus* species	insecticidal activity	Birkett *et al.*, 2000; Jabalpurwala *et al.*, 2009.
59	nerolidol	sesquiterpene	*Citrus* species	insecticidal activity	Lota *et al.*, 2002; Arruda *et al.*, 2005; Jabalpurwala *et al.*, 2009.
60	thymol	monoterpene phenol	*Citrus* species; *Origanum vulgare*	antimicrobial	Jabalpurwala *et al.*, 2009; Daferera *et al.*, 2000; Daferera *et al.*, 2003; Marino *et al.*, 2001.

61	n-phenylformamide		*Citrus* species		
62	aromadendrene	sesquiterpene	*Citrus* species	antifungal	Hammer *et al.*, 2003; Hosni *et al.*, 2010.
63	α-terpinol	monoterpene alcohol	*Citrus limon*	antifungal	Lota *et al.*, 2002; Hammer *et al.*, 2003.
64	α-cyperone	sesquiterpene	*Citrus* species		Hosni *et al.*, 2010.
65	geranyl-α-terpinene		*Citrus* species		Hosni *et al.*, 2010.
66	δ-cadinene	sesquiterpene	*Citrus* species		Hosni *et al.*, 2010.
67	germacrene-B	sesquiterpene hydrocarbon	*Citrus* species		Hosni *et al.*, 2010.
68	germacrene-D	sesquiterpene hydrocarbon	*Citrus* species	insecticidal activity	Lota *et al.*, 2002; Røstelien *et al.*, 2000; Hosni *et al.*, 2010.
69	α-sinensal	sesquiterpene aldehyde	*Citrus* species		Lota *et al.*, 2002; Hosni *et al.*, 2010.
70	β-sinensal	sesquiterpene aldehyde	*Citrus* species		Lota *et al.*, 2002; Røstelien *et al.*, 2000; Hosni *et al.*, 2010.
71	γ-eudesmol	sesquiterpenoid	*Citrus* species		Hosni *et al.*, 2010.
72	τ-cadinol	sesquiterpene	*Citrus* species		Hosni *et al.*, 2010.
73	bicyclogermacrene	sesquiterpene hydrocarbon	*Citrus* species		Hosni *et al.*, 2010.
74	caryophyllene oxide	sesquiterpene oxide	*Citrus limon*	antifungal activity	Lota *et al.*, 2002; Yang *et al.*, 2000.
75	α-caracorene	sesquiterpene	*Citrus* species	anti-inflammatory	Chavan *et al.*, 2010; Hosni *et al.*, 2010.
76	Eugenol	phenol	*Syzygium aromaticum* (clove)	antibacterial	Bauer *et al.*, 2001.
77	Eugenyl acetate		*Syzygium aromaticum* (clove)	antibacterial	Bauer *et al.*, 2001.

4. Fungal spoilage of food

Food are susceptible to many different contamination microorganisms (bacteria, fungi and enteric virus) and sources, such as seed, soil, irrigation water, animals, manure/sewage sludge use, harvesting, processing and packaging. Generally, the kind of spoilage depends from the composition of food and the proliferation of these microorganisms can lead to food losses (defects of texture and off-odors due to enzymes and metabolites release) or to human diseases (in case of contamination with pathogenic microorganisms and/or their toxins).

In this context, food decay by spoilage fungi causes considerable economic losses and constitutes a health risk for consumers due to the potential for fungi to produce mycotoxins. The indiscriminate use of synthetic antifungals has led to the development of resistant strains, which need higher concentrations of compounds to be killed, with the consequent increase of toxic residues into food products.

Molds are a large group of taxonomically diverse fungal species, which are able to colonize opportunistically a wide array of habitats including foods, especially fresh fruits, vegetables, and grains. Because of the high activity of their hydrolytic enzymes and the production of toxic metabolites such as mycotoxins, molds are responsible for the decay or deterioration of a wide variety of foods and cause quantitative and qualitative losses. Worldwide, post-harvest losses have been estimated at 50% and much of this is due to fungal and bacterial infections (Magro *et al.*, 2006). Fruits and vegetables are highly susceptible to fungal spoilage, both in the field and during postharvest storage. Significant spoilage fungi genera include *Pythium, Phytophthora, Aspergillus, Fusarium, Penicillium, Alternaria, Botrytis,*

Geotrichum, *Sclerotinia* and *Rhizoctonia* spp. Mold growth mainly depends on abiotic factors such as pH, water activity (aw), solute concentration, temperature, atmosphere, time, etc. Although, the main variables determining the development of fungi are the temperature and the aw.

In addition, many species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* can synthesize mycotoxins, hazardous compounds since they are carcinogenic, mutagenic, teratogenic and immunosuppressant. Their activity depends on the type of toxin and their concentration in the food. Concern about these chemical hazards has been increasing due to the wide range of food types that may be affected and the variability in the severity of symptoms caused. Mycotoxins can be produced before and after harvest and levels may increase during postharvest handling and storage. Thus, prevention of fungal growth is an effect means of preventing mycotoxin accumulation. Mycotoxins may reach consumers either by direct contamination of plant materials or products thereof, or by 'carry over' of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed. Furthermore, this hazard remains in processed food because these metabolites are not removed by standard industrial processing, and the risk could increase if moldy fruits or plants are used in processed byproducts.

5. Pathogenic microorganisms in food

Pathogens may be present on food originating from the raw materials or due to cross-contamination during processing (Nguyen-the *et al.*, 1994; Beuchat, 1996; Seymour

et al., 2001). The incidence of foodborne outbreaks caused by contaminated food has increased in recent years (WHO, 2002; EFSA, 2015; Mukherjee *et al.*, 2006).

The pathogens most frequently linked to produce-related outbreaks include bacteria (*Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*), enteric viruses (noroviruses, hepatitis A virus), and parasites (*Cryptosporidium*, *Cyclospora*) (Tauxe *et al.*, 1997), with *Salmonella* being the leading cause in the EU (EFSA, 2015). Fresh produce and sprouts have been implicated in a number of documented outbreaks of illness in countries such as Japan (Nat'l. Inst. Inf. Dis., 1997), USA (De Roever, 1998) and EU (Emberland *et al.*, 2007; Pezzoli *et al.*, 2007; Abadias *et al.*, 2008; Söderström *et al.*, 2005; Rasko *et al.*, 2011).

Many pathogens have been isolated from different kind of food, although not all of them could be directly associated with foodborne outbreaks. The most important bacterial foodborne pathogens are discussed below.

Salmonella species

Salmonella is a rod Gram-negative, facultative anaerobic, mobile and nonspore forming bacteria. It is mesophilic fecal-associated pathogen. Its presence is mostly associated to meat, milk and dairy products, fish and fresh-cut vegetables and its growth on these products is generally associated to temperature abuse (T>10°C). On the contrary, the low pH is a limit for the pathogen growth. This pathogen has a very low infectious dose of less than 100 cells. *Salmonella* is frequently present on raw vegetables and fruits (Doyle, 1990; Beuchat, 1996, Abadias *et al.*, 2008). Normally its growth rate is reduced at less than 15°C and prevented at less than 7°C (ICMSF,

1996). Studies of fresh, unprocessed produce conducted in Minnesota and Wisconsin (Mukherjee *et al.*, 2006), UK (Sagoo *et al.*, 2003), in southern USA (Johnston *et al.*, 2005) and in USA with imported fresh produce (FDA, 2001) and Malaysia showed widely varying incidences of *Salmonella*: 0, 0.2%, 3.3%, 3.5% and 35%, respectively.

Refrigeration is the best preservation method to prevent an outgrowth of this mesophilic pathogen. In the European regulation regarding criteria for foodstuffs (EU Regulation 2073/2005), it is generally recommended the absence of *Salmonella* in 25 g of product.

Escherichia coli

E. coli is a rod Gram-negative, mesophilic, facultative anaerobic, non-sporigen fecal-associated pathogen. It belongs to *Enterobacteriaceae* family and it is commonly found in the intestines of warm-blooded animals (as further described in the paragraph below). Most types of *E. coli* are harmless, but some are pathogenic, being enterovirulent (EEC). The symptoms of *E. coli* O157:H7 infection, an enterohemorrhagic strain, include severe, sometimes bloody, diarrhea and abdominal cramps. The number of *E. coli* O157:H7 infections associated to food have increased in the last years (EFSA, 2015). Survival and growth patterns of *E. coli* O157:H7 are dependent on food type, package atmosphere and storage temperature (Francis *et al.*, 2001). Meat and cured meats, raw milk and fruit juices are commonly associated to this pathogen. *E. coli* can be present in raw material and in vegetables and fruits. *E. coli* has the capability to grow at high temperatures and to survive at refrigerated

temperatures. In addition, its low infectious dose (10 to 100 CFU/g) makes the presence of this pathogen a risk for public health (Chang *et al.*, 2007). *E.coli* is used as hygiene indicator since it is a fecal-associated bacteria and its presence is linked to the possible presence of other fecal pathogens (Ragaert *et al.*, 2011), such as *L. monocytogenes*.

Listeria monocytogenes

L. monocytogenes is a rod Gram-positive, non-sporigen, facultative anaerobic pathogen. It is widely distributed in natural environment including foodstuffs as, milk and dairy products, fermented and raw meat, fish, refrigerated food and raw vegetables (Carlin *et al.*, 1994; Koseki *et al.*, 2005; Beuchat, 1996). Its minimal growth temperatures are between 0 and 4°C, it is not affected by modified atmospheres applied for meat, fish, fresh-cut vegetables and fruits (Thomas *et al.*, 1999; Rodriguez *et al.*, 2000). In Europe, the reported hospitalisation and case-fatality rates due to *L. monocytogenes* in confirmed human cases accounted for 0.56% of all the zoonoses registered in 2013 (EFSA, 2015). These data show an increasing trend of listeriosis in the EU over the period 2009-2013.

As food safety criteria for ready-to-eat foods intended for infants and for special medical purposes the EU Regulation 2073/2005 establishes the absence of *L. monocytogenes* in 25 g of products (following the analytical reference method EN/ISO 11290-1). For ready-to-eat foods able to support the growth of the pathogen, the limit is 100 cfu/g for products placed on the market during their shelf-life, while the absence in 25 g is established before the food has left the immediate control of

the food business operator, who has produced it. For ready-to-eat foods unable to support the growth the limit is 100 cfu/g.

Outside Europe, there are often different criteria regarding the presence of *L. monocytogenes* in food. For example, USA and Canada introduced a zero tolerance for some foods (absence of *L. monocytogenes* in 25 g), especially foods that are supportive of growth and have extended shelf-life. In these countries, decontamination techniques are often allowed in the production chain in order to reduce the bacterial load and avoid the presence of pathogens.

Staphylococcus aureus

S. aureus is a Gram-positive, facultative anaerobic, coccal bacterium frequently found in the respiratory tract and on the skin. It is mesophilic, not mobile and not sporigen.

Although *S. aureus* is not always pathogenic since it may occur as a commensal, it is a common cause of skin infections and food poisoning. Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant forms of *S. aureus* such as MRSA (methicillin-resistant *Staphylococcus aureus*) is a worldwide problem in clinical field.

The intoxication from contaminated food is due to its toxins. More than 60% of strains result enterotoxigenic producing enterotoxins. In those cases the severity of illness is related to the amount of toxin taken in. It is often associated to meat products, eggs, tuna, vegetables, milk and cheeses.

Enterobacteriaceae

The *Enterobacteriaceae* is a family of common Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria. The strains related to food contaminations may be pathogenic, opportunistic pathogens or hygiene and safety indicators. Since they are a normal part of the gut microbiota found in the intestines of humans and other animals, they can contaminate almost all kind of food matrices. In meat and fish, their decarbossilation activity could result in biogenic ammine production as putrescine, cadaverine and histamine. Common species that belong to *Enterobacteriaceae* family are *Enterobacter aerogenes*, *E. cloacae*, *E. agglomerans*, *Escherichia coli*, *Citrobacter freundii*, *Erwinia erbicola*, *Erwinia carotovora*, *Serratia* spp. and *Proteus* spp.

6. Tests to evaluate the antibacterial and antifungal activity of EOs

In vitro tests to evaluate the antibacterial and antifungal activity of compunds can be classified as diffusion, dilution, vapor phase or bioautographic methods (Rios *et al.*, 1988). So far standardized test has not been developed at least for evaluating the efficacy of natural compounds, such EOs. The NCCLS method (NCCLS, 2000) for antibacterial susceptibility testing, which is principally aimed at the evaluation of antibiotics for clinical purposes, was modified for the evaluation of EOs (Hammer *et al.*, 1999). Moreover, researchers adapt experimental methods to better represent possible future applications in their particular field. However, since many factors may affect the result of the test, it is recommended to specify the extraction method

of EO, the plant material, the volume of inoculum, the culture medium used and the incubation time and temperature (Rios *et al.*, 1988). All these variables really complicates comparisons among published studies (Friedman *et al.*, 2002; Janssen *et al.*, 1987). Generally, a preliminary screening of EOs for antibacterial and antifungal activity is often done by the disk diffusion method, in which a paper disk soaked with EO is laid on top of an inoculated agar plate. The well diffusion method is a similar test in which EOs are added into wells performed directly into the agar layer of the plate.

Since EOs are constituted by volatile compounds, a vapor phase test is used to assess their antimicrobial activity. In this case, seeded plates are left to incubate upside down with a paper disk spotted with EO put onto the upper lid of the Petri dish. In all of these tests, the generated inhibition zone (or halo) is considered a criterion to evaluate the antimicrobial activity. Another test widely used for antifungal activity evaluations is the poisoned food technique. The fungicidal action is expressed in terms of percentage of mycelia growth inhibition respect to an untreated control. Generally, the results are picked up by the evaluation of the growth of the organism by visual inspection, by measuring the optical density (OD) or by viable counts.

The minimum inhibitory concentration (MIC) is cited by most researchers as a measure of the antibacterial performance of EOs, although many definitions have been indicated. MIC is referred to as: (i) the lowest concentration resulting in maintenance or reduction of inoculum viability (Carson *et al.*, 1995); (ii) the lowest concentration required for the complete inhibition of test organism up to 48 h incubation (Canillac *et al.*, 2001; Wan *et al.*, 1998); (iii) the lowest concentration

inhibiting visible growth of test organism (Delaquis *et al.*, 2002; Hammer *et al.*, 1999; Karapinar *et al.*, 1987; Onawunmi, 1989); (iv) the lowest concentration resulting in a significant decrease in inoculum viability (>90%) (Cosentino *et al.*, 1999). Others terms used for testing antimicrobial activity are: minimum bactericidal concentration (MBC) defined as the concentration where 99.9% or more of the initial inoculum is killed (Canillac *et al.*, 2001; Carson *et al.*, 1995; Cosentino *et al.*, 1999) or as the lowest concentration at which no growth is observed after sub culturing into fresh broth (Onawunmi, 1989); bacteriostatic concentration cited as the lowest concentration at which bacteria fail to grow in broth, but are cultured when broth is plated onto agar (Smith-Palmer *et al.*, 1998); bactericidal concentration used to indicate the lowest concentration at which bacteria fail to grow in broth, and are not cultured when broth is plated onto agar. Viability assays, such as time-kill analysis, are commonly used to assess the rapidity of an antimicrobial effect or the duration of a bacteriostatic effect. The result of viable cells is expressed by plotting viable cells remaining in broth after a defined contact time with an EO against time (survival curve plot).

Nowadays, molecular methods are available to rapidly and accurately detect viable and dead cells after a given treatment, including EOs exposure. When applied in food industry, these PCR-based techniques can be a usefull tool to control and prevent pathogen contaminations (O'Grady *et al.*, 2009). In fact, it is possible to detect bacteria in different food matrices and the results can be obtained more rapidly comparing with the standard culture methods (De Boer *et al.*, 1999; Malorny *et al.*, 2003). This is particularly important for minimally processed vegetable and fruit

since they enjoy a short shelf-life. In addition, the evaluations by using traditional culture methods show the lack of sensitivity regarding viable but not culturable cells (VBNC) (Randazzo *et al.*, 2016; Kramer *et al.*, 2009).

The main drawback when applying PCR for pathogen detection in food is how to distinguish between DNA from dead and live cells (Rudi *et al.*, 2002). In fact, DNA from dead cells, killed by processing procedures or other factors such as EO, can serve as a template during PCR amplification (Nogva *et al.*, 2003). This is particularly relevant for processed foods resulting in false positive results. A promising strategy to avoid this issue relies on the use of nucleic acid intercalating dyes, such as propidium monoazide (PMA) or ethidium monoazide (EMA) as a sample pre-treatment before the qPCR. This procedure is based on the integrity of bacterial cells since these dyes penetrate only into damaged membrane or dead cells (Nocker *et al.*, 2006). PMA proved to be more selective compared to EMA because of the higher charge of the molecule (Nocker *et al.*, 2006) and only penetrates into membrane-compromised or dead cells. PMA action is based on the presence of an azide group that allows cross-linking of the dye to DNA after exposure to strong visible light. The light leads to the formation of a highly reactive nitrene radical that strongly inhibits DNA amplification. PMA treatment combined with qPCR has been successfully tested on bacterial pathogens such as *L. monocytogenes* (Pan *et al.*, 2007), *E. coli* O157:H7 (Elizaquível *et al.*, 2011; Nocker *et al.*, 2009) and *Campylobacter jejuni* (Josefsen *et al.*, 2010) among others.

Regarding the tests assessed for evaluating the antimicrobial activity of EOs in food, viable direct counts of intentionally contaminated food after EO addiction/treatment

have been widely used. It is generally supposed that higher amount of EOs are needed in food to achieve results similar to the *in vitro* tests. This could be explained by the lesser efficacy of EOs in damaging microbial cells and their faster ability in self-repairing. Indeed, the greater availability of nutrients in foods compared to laboratory media may enable bacteria to better repair damaged cells (Gill *et al.*, 2002). On the other hand, the intrinsic properties of the food (fat, protein, water content, antioxidants, preservatives, pH, salt and other additives) can influence both the microbial sensitivity (Shelef, 1983; Tassou *et al.*, 1995) and the efficacy of EO, due to its lower availability. For example, at low pH the hydrophobicity of an EO increases, enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria (Juven *et al.*, 1994). As well, high levels of fat and/or protein in foodstuffs protect the bacteria from the action of the EO (Aureli *et al.*, 1992; Pandit *et al.*, 1994; Tassou *et al.*, 1995). The physical structure of a food may also limit the antimicrobial activity of EO due to the limitation of diffusion (Skandamis *et al.*, 2000a). The extrinsic characteristics of a food, such as temperature, packaging system (in vacuum, gas, air) and traits of microorganisms also affect EOs activity. For example, the antimicrobial activity of EOs in vegetables increases with a decrease in storage temperature (Skandamis *et al.*, 2000b).

7. Mode of antibacterial and antifungal action

The mechanism of action of EOs has not been elucidated in detail (Lambert *et al.*, 2001). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial and antifungal activity is not

attributable to one specific mechanism but that there are several targets in the cell (Carson *et al.*, 2002; Skandamis *et al.*, 2001). Anyway, all the mechanisms involved in bacterial cells inhibition by EOs can be summarized as the degradation of the cell wall (Helander *et al.*, 1998; Thoroski *et al.*, 1989); the damage of cytoplasmic membrane (Knobloch *et al.*, 1989; Oosterhaven *et al.*, 1995; Sikkema *et al.*, 1994; Ultee *et al.*, 2002; Ultee *et al.*, 2000); the damage of membrane proteins (Juven *et al.*, 1994; Ultee *et al.*, 1999); the leakage of cell contents (Cox *et al.*, 2000; Gustafson *et al.*, 1998; Helander *et al.*, 1998; Lambert *et al.*, 2001; Oosterhaven *et al.*, 1995); the coagulation of cytoplasm (Gustafson *et al.*, 1998) and the depletion of the proton motive force (Ultee *et al.*, 1999; Ultee *et al.*, 2001). Some of these targets are affected because of another mechanism being targeted.

An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the cell membrane, disturbing the structures and rendering them more permeable (Knobloch *et al.*, 1986; Sikkema *et al.*, 1994). Leakage of ions and other cell contents can then occur (Cox *et al.*, 2000; Gustafson *et al.*, 1998; Helander *et al.*, 1998; Lambert *et al.*, 2001; Oosterhaven *et al.*, 1995; Skandamis *et al.*, 2001; Ultee *et al.*, 2002).

Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer *et al.*, 1991). There is some evidence from studies with tea tree oil and *E. coli* that cell death may occur before lysis (Gustafson *et al.*, 1998).

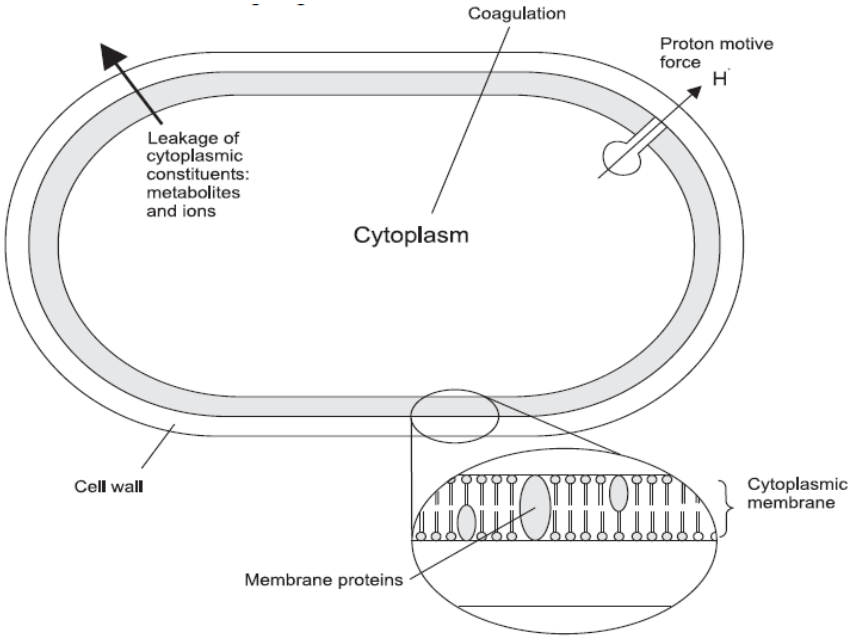


Figure 3. Sites of action of essential oils or their compounds at the bacteria cellular level: cell wall degradation; damage of the cytoplasmic membrane; damage of membrane proteins; loss of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Burt, 2004).

EOs are slightly more active against Gram-positive than Gram-negative bacteria (Canillac *et al.*, 2001; Cimanga *et al.*, 2002; Delaquis *et al.*, 2002; Lambert *et al.*, 2001; Pintore *et al.*, 2002; Settanni *et al.*, 2014). Gram-negative organisms are less susceptible to the action of antibacterials since they possess an outer membrane surrounding the cell wall (Ratledge *et al.*, 1988) which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992). However, not all studies on EOs have concluded that Gram-positives are more susceptible (Wilkinson *et al.*, 2003).

The chemical structure of the individual EO components affects their precise mode of action and its antibacterial activity (Dorman *et al.*, 2000). The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed (Dorman *et al.*, 2000; Knobloch *et al.*, 1986; Ultee *et al.*, 2002).

Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Juven *et al.*, 1994). Carvacrol and thymol appear to make the cell membrane permeable (Lambert *et al.*, 2001) and both compounds are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP.

The biological precursor of carvacrol, p-cymene is hydrophobic and causes swelling of the cytoplasmic membrane to a greater extent than does carvacrol (Ultee *et al.*, 2002). p-Cymene is not an effective antibacterial when used alone (Dorman *et al.*, 2000, Juven *et al.*, 1994; Ultee *et al.*, 2000), but when combined with carvacrol, synergism has been observed against *B. cereus* in vitro and in rice (Ultee *et al.*, 2000). Although cinnamaldehyde (3-phenyl-2-propenal) is known to be inhibitive to growth of *E. coli* O157:H7 and *S. typhimurium* at similar concentrations to carvacrol and thymol, it did not disintegrate the outer membrane or deplete the intracellular ATP pool (Helander *et al.*, 1998). The carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (Wendakoon *et al.*, 1995).

Regarding the mechanism of action of EOs against fungal species, it still remains poorly understood due to the difficulties already summarized by Lanciotti *et al.* (2004) and Prudent *et al.* (1995). As occur for bacteria cells, it has suggested that the cell membrane is the possible target of bioactive volatile compounds because of the fact that EOs are mixtures of molecules characterized by their poor solubility in

water and high hydrophobicity (Akgül *et al.*, 1988; Kim *et al.*, 1995; Lattaoui *et al.*, 1994).

Several studies have showed that terpenes and phenolic compounds can disrupt the membrane of both fungi and bacteria (Ratledge *et al.*, 1988; Rees *et al.*, 1995).

Monoterpenes act by disrupting the microbial cytoplasmic membrane, resulting in the loss of membrane impermeability. If the disturbance of membrane integrity occurs, then its functions are compromised not only as a barrier but also as a matrix for enzymes and as an energy transducer (Rees *et al.*, 1995; Renzini *et al.*, 1999).

In a more recent study by Tao *et al.* (2014), the antifungal activity of mandarin EOs against *P. italicum* and *P. digitatum* was attributed to the monoterpenes in the oils, such as limonene, octanal and citral. They also suggested that mandarin EOs generated cytotoxicity by disrupting cell membrane integrity, causing the leakage of cell components.

Concluding, the antifungal mechanism of action of the bioactive compounds of EOs can be explained by the same mechanism of action of bacterial one.

8. Organoleptic, safety and legal aspects of the use of EOs and their components in foods

The application of EOs in food may result in a strong organoleptic impact depending on the doses applied. Since the antimicrobial activity of EOs have been shown to be dose-dependent, the amount to be applied in a specific food to obtain a desired antimicrobial effect can vary at a large extent, finally resulting in altering organoleptic profile of food when high doses are needed. In fact, the reduction of the

doses to be applied to food matrixes is the clue to be pursued to apply EOs extensively. While some foods are already associated by consumers with herbs and spices giving a defined and pleasant aroma (e.g. fish and meat), some others are not, such as the case of fruits or vegetables. In all cases when EOs are added to food, sensory analyses must be performed to ascertain their effects on the sensory characteristics of food. Many studies support the final acceptability of meat (Tsigarida *et al.*, 2000; Skandamis *et al.*, 2001) and fish (Mejlholm *et al.*, 2002; Harpaz *et al.*, 2003) preparations added with EOs, even if differences are usually detected by the panel test. In addition, EO compounds have been used to treat fruits such as kiwifruit and honeydew melon without causing adverse organoleptic changes (Roller *et al.*, 2002).

A rising trend to avoid the direct organoleptic impact of EOs, moreover associated with their prolonged dispersion and longer antimicrobial effect, is their inclusion in food packaging materials. As a matter of facts, the use of edible coatings as carriers of antimicrobial compounds could be an alternative tool to combact food spoilage and/or pathogen agents (Aider, 2010; Bakkali *et al.*, 2008; Burt, 2004; Sánchez-González *et al.*, 2011) and, at the same time, to reduce the amount of EOs to be applied in food. In this way, the chemico-physical properties of the polymer constituting the film and acting as a selective barrier to gas transport (Vargas *et al.*, 2008), together with the antimicrobial properties of EOs included, can be the goal of an hurdle technology applied to food to extend its commercial shelf-life (Park, 1999; Perdones, *et al.*, 2012).

All the above considerations must take as well into account the irritation and toxicity of EOs to eukaryotic cells, and then to human epithelia. Even if some EOs are considerate as GRAS and approved as food flavourings, cytotoxicity studies showed that eugenol, menthol and thymol, applied in root canal treatments caused irritation of mouth tissues (Manabe *et al.*, 1987). Moreover, they may cause spasmogenic effects (Lis-Balchin *et al.*, 1999) or allergic contact dermatitis in people who use them frequently (Bleasel *et al.*, 2002; Carson *et al.*, 2001). Then, more safety studies should be carried out before EOs become more widely used or at greater concentrations in foods that at present.

In contrast, a number of EO components have been registered by the European Commission for use as flavourings in foodstuffs. The flavourings registered are considered to present no risk to the health of the consumer and include amongst others carvacrol, carvone, cinnamaldehyde, citral, *p*-cymene, eugenol, limonene, menthol and thymol. The EU registered flavourings listed above also appear on the 'Everything Added to Food in the US' (EAFUS) list (http://www.fda.gov/), which means that the United States Food and Drug Administration (FDA) has classified the substances as GRAS or as approved food additives.

In conclusion, the approval of EOs as food additives should involve a deeper knowledge of their antimicrobial effect, as well as their biological toxicity against gastro-intestinal cells and, finally, their impact onto the sensorial food profile.

The aims of the Ph.D.

research thesis

The aims of the Ph.D. research thesis

This research work has been developed in the context of the potencial applications of essential oils as multi-target compounds for novel food safety strategies, such as active packaging technologies.

Essential oils are aromatic oils derived from plants and usually extracted by hydrodistillation when intended to be used in food. There is an increasing interest in the antimicrobial properties of essential oils due to the possibility of using them to replace 'synthetic' preservatives in food or, in general, to reduce viable numbers of pathogens along the food chain.

With this perspective, **the first aim** of the present research thesis was to study the antibacterial effectiveness of *Citrus* EOs against common pathogens associated to foodborne outbreaks (*Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica, Enterobacter* spp., *Escherichia coli*) (Chapter 1; Chapter 2; Chapter 3; Chapter 4). Moreover, the influence of the harvesting stages on both chemical compositions and antimicrobial activity has also been taken into account (Chapter 1). Since strain-dependent responses to EOs exposure have been reported, a further study has been carried out to evaluate the effect of *Citrus* EOs against a collection of *Listeria monocytogenes* strains and, thereafter, the antimicrobial properties have been assessed in different edible film formulations (chitosan and methylcellulose coatings) (Chapter 2).

Nowadays, the pathogen detection can be rapidly evaluated by applying PCR techniques, but the lack in distinguishing between DNA from dead and live cells is one of the mayor drawbacks of these techniques. A promising strategy to avoid this

issue relies on the use of DNA intercalating dyes, such as propidium monoazide (PMA) as a sample pretreatment previous to the real-time PCR. On the base of these considerations, the **second aim** of this research was the evaluation of the applicability of PMA-qPCR technique for the detection and quantification of viable *E. coli* O157:H7 cells after inactivation by *Citrus* EOs (Chapter 4).

The **third aim** of this research thesis focused on the determination of the antifungal activity of the *Citrus* EOs, previously assessed for their antibacterial activities. The inhibition has been evaluated against forty-seven foodborne filamentous fungal strains of different species and origin. Furthermore, for these experiments activity of the EOs has been correlated to their chemical composition (Chapter 5).

Chapter 1

Seasonal variations of antimicrobial activity and chemical composition of essential oils extracted from three Citrus limon L. Burm. cultivars

1.1. Materials and Methods

1.1.1. *Citrus* samples and EOs extraction

The EOs analyzed in this study were obtained from peels of the following three cultivars of *Citrus limon* L. Burm.: Femminello Santa Teresa, Monachello and Femminello Continella, selected as the best producers of inhibition activity among previously tested *Citrus* EOs (Settanni *et al.*, 2012).

Lemon fruits were collected in the orchard "Parco d'Orleans" of the Agricultural and Forest Science Department – University of Palermo (Palermo, Italy) at 6-week intervals for a total of 4 harvests (A, December 2012; B, January 2013; C, February 2013; D, April 2013). After the harvest, lemon peels were immediately subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Comandè, Palermo, Italy) collecting the oil in hexane. EOs were dried over anhydrous sodium sulphate and stored at 4°C in air-tight sealed glass vials covered with aluminum foil prior to be used in chemical and microbiological analysis.

1.1.2. Bacterial strains

Ninety-eight pathogen strains were tested for their sensitivity to EOs. The strains were provided by the Department of Sciences for Health Promotion and Mother-Child Care "G. D'Alessandro" – University of Palermo (Palermo, Italy) and belonged to *Listeria monocytogenes* (20 strains; Table 1.1), *Staphylococcus aureus* (26 strains; Table 1.2), *Salmonella enterica* (14 strains; Table 1.3) and *Enterobacter* spp. (38 strains; Table 1.4). Bacterial strains were sub-cultured in Brain Heart Infusion (BHI) agar (Oxoid, Milan, Italy) and incubated overnight at 37°C.

1.1.3. Evaluation of the antibacterial activity

A modified paper disc diffusion method (Militello *et al.*, 2011) was applied to test the antibacterial activity of the lemon EOs. Bacterial cells were grown overnight before tests. The cells were centrifuged at 10.000 × g for 5 min, washed in Ringer's solution (Sigma-Aldrich, Milan, Italy) and re-suspended in the same solution until the optical density (OD) of ca. 1.00, measured by 6400 Spectrophotometer (Jenway Ltd. Felsted Dunmow, UK) at 600 nm, which approximately corresponds to a concentration of 10^9 CFU ml^{-1} as estimated by plate count in Nutrient Agar (NA) incubated 24 h at 28°C. A final concentration of approximately 10^7 CFU ml^{-1} of each strain was inoculated into 7 ml of BHI soft agar (0.7% w/v) and poured onto NA.

Sterile filter paper discs (Whatman No. 1) of 6 mm diameter were placed onto the surface of the double agar layer and soaked with 10 µL of EO. Sterile water and streptomycin (10%, w/v) were used as negative and positive control, respectively. Incubation was at 37°C for 24 h. Antibacterial activity was scored positive when a definite halo of inhibition, whose width could be clearly measured, was detected around the paper disc. Each test was performed in duplicate and the experiments were repeated twice in two consecutive days.

1.1.4. EOs chemical composition

For analysis of the EOs samples, 1.0 ml of solution (1:10 v/v, essential oil/hexane) was placed in injection port. The extraction of volatile aroma compound was carried out using the gas chromatography/mass spectrometry (GC/MS) (EI) on a GCMS-QP2010 (Shimadzu, Milan, Italy).

GC condition: Gas chromatography equipped with a fused silica capillary column SLB-5MS (5% diphenyl:95% methylsiloxane) 30 m x 0.25 i.d. x 0.25 mm film thickness (Supelco, Milan, Italy); carrier gas He at a constant linear rate 30 cm s^{-1} (30.6 kPa); split/splitless injector port; injector temperature 250°C; injection mode split (split ratio 100:1). The oven temperature program: 50°C, hold 3 min^{-1}; 3°C min^{-1} to 240°C; 15°C min^{-1} to 280°C, hold 1 min. MS scan conditions: source temperature 200°C, interface temperature 250°C, EI energy 70 eV; mass scan range 40-400 amu. Data were handled through the use of GCMS-Solution software and the peak identification was carried out with NIST21,107,147 Library according to a similarity larger than 90% and other published mass spectra. Identification of components was confirmed by comparison of experimental linear retention indexes with those available in literature. GC/MS analysis was carried out in duplicate.

1.1.5. Statistical analysis

Data of inhibitory activities and concentration of chemicals were statistically analysed using the generalised linear model (GLM) procedure, including the effects of EO and ripening stage and their interaction, with the program SAS 2008 – version 9.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). The Student "t" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison.

1.2. Results and Discussion

1.2.1. Inhibition of bacterial growth

In this study, the inhibitory spectra of the EOs extracted from the peel of the fruits of three cultivars of *Citrus limon* L. Burm. harvested at four times were evaluated against 98 strains of *S. aureus*, *L. monocytogenes*, *Salmonella enterica* and *Enterobacter* spp. of different origin which are reported to be responsible for human diseases commonly associated with the consumption of contaminated food items (Wilson *et al.*, 2000; Swaminathan *et al.*, 2007; Crum-Cianflone, 2008; Healy *et al.*, 2010). They may contaminate the final foods starting from the raw materials or during manufacturing and/or during storage and handling steps (Adams *et al.*, 2002). In particular, *S. aureus* has been reported as the most common pathogen isolated from domestic refrigerators (Jackson *et al.*, 2007). For these reasons, it is evident the importance of keeping the growth of the above species under control.

Inhibitory activities of lemon EOs against the food-borne pathogens are reported in Tables 1.1-1.4. In general, the different cultivars and the different times of lemon collection affected significantly the inhibitory efficacy of EOs and the statistical differences were often consistent ($P \leq 0.001$). However, this behavior was not observed against *L. monocytogenes* 135 and 14BO, *S. aureus* E36GIMRSA, *E. hormaechei* 13, *E. cloacae* 24 and 32A and *Enterobacter* spp. 8UTIN ($P > 0.05$).

The inhibitory power of EOs was found to be strain-dependent because the differences registered among the different strains were statistically significant. It was not directly correlated with the lemon maturation for some strains ($P > 0.05$), but, on the contrary, it increased with time for some others ($P \leq 0.05$ – $P \leq 0.001$). Furthermore,

the intermediate harvests of lemons (January and February) did not show antibacterial activity of the resulting EOs for some strains, while an opposite trend was observed *vs* other strains which were inhibited only by the EOs of the intermediate harvests.

Femminello Santa Teresa EO inhibited all *L. monocytogenes* except the strains 1BO in the February production, the strain 20BO in December and the strain 24BO in January and February (Table 1.1). *L. monocytogenes* 1BO was not inhibited even by the February production of Monachello and Femminello Continella EOs. The EO showing the lowest efficacy, both in terms of number of strains inhibited and diameter of the inhibition halos, against *L. monocytogenes* was that extracted from the cultivar Femminello Continella.

Almost all strains of *S. aureus* were inhibited by EOs of Femminello Santa Teresa and Monachello at the different times of harvest, while several strains were not inhibited ($P>0.05$) by at least one harvest of Femminello Continella (Table 1.2).

Within the Gram-negative strains, *Salmonella* (Table 1.3) showed a lowest sensitivity than *Enterobacter* (Table 1.4). Also against these strains, EO from the cultivar Femminello Continella showed a lesser activity than the other two EOs at the different harvest stages. However, the inhibition areas detected for several *Enterobacter* isolates were significantly larger ($P\leq0.05$ – $P\leq 0.001$) than those measured for *L. monocytogenes* and *S. aureus*. All *E. hormaechei* isolates were inhibited by the EO of all harvests of the cultivar Femminello Santa Teresa.

All EOs tested were more effective, in terms of percentage of the strains inhibited, against the Gram-positive rather than Gram-negative bacteria. This finding is not surprising, since also other studies showed that Gram-positive bacteria were more

susceptible to EOs of different origin, including citrus, than Gram-negative bacteria (Davidson *et al.*, 2000; Burt, 2004; Calsamiglia *et al.*, 2007; Al-Reza *et al.*, 2010). These findings have been related to the presence of the outer membrane in Gram-negative bacteria, which provides a strong impermeable barrier (Nikaido, 1994).

Table 1.1. Inhibitory activity[a] of lemon EOs extracted from fruits harvested at different collection times[b] against *Listeria monocytogenes.*

Strain	*Citrus limon* L. Burm. spp. cultivars												SEM	Statistical significance[a]		
	Femminello Santa Teresa				Monachello				Femminello Continella							
	A	B	C	D	A	B	C	D	A	B	C	D		CV	CT	CV*CT
129	1.00	0.90	0.90	0.90	0.75	0.90	1.30	0.90	0.00	0.00	0.95	0.85	0.08	***	***	***
133	0.90	0.80	1.05	0.75	0.85	1.10	1.50	0.85	0.00	0.75	1.10	0.70	0.07	***	***	***
134	1.05	0.90	1.20	1.10	1.20	0.95	1.20	1.05	0.90	0.90	0.00	0.95	0.07	***	***	***
135	0.90	0.80	1.05	0.70	1.10	0.90	0.90	0.70	0.85	0.75	0.85	0.80	0.08	ns	ns	ns
140	1.05	0.70	0.95	0.90	0.85	0.85	1.30	1.00	0.00	0.75	1.05	0.70	0.07	***	***	***
180	1.15	0.95	1.00	0.95	0.75	1.10	1.50	0.70	0.70	0.85	0.95	0.90	0.07	*	**	***
182	0.95	0.95	1.65	1.10	0.80	1.20	1.70	0.80	0.00	0.85	1.00	0.85	0.05	***	***	***
184	0.95	1.05	1.00	1.20	0.85	1.00	1.10	1.25	0.85	0.75	0.70	1.00	0.05	***	***	ns
185	0.85	1.05	1.20	1.10	0.00	0.90	1.40	1.20	0.00	0.90	1.00	1.15	0.06	***	***	***
186	1.45	0.75	1.10	1.10	0.80	0.90	1.25	0.90	0.80	0.70	0.90	0.90	0.08	**	**	**
187	1.30	0.85	1.15	0.90	0.90	0.70	1.10	1.00	0.80	0.85	0.85	0.80	0.06	**	**	**
188	0.95	0.75	0.70	0.90	0.75	0.90	0.75	0.90	0.85	0.90	0.00	0.90	0.90	*	***	***
1 BO	1.05	1.05	0.00	0.90	0.85	0.85	0.00	0.80	0.80	0.80	0.00	0.90	0.05	**	***	ns
3 BO	1.35	1.10	0.85	1.00	0.75	0.90	0.95	1.05	0.80	0.80	0.90	0.00	0.07	***	***	***
4 BO	0.85	1.10	1.25	0.90	0.00	0.70	1.35	0.95	0.90	0.00	0.95	0.80	0.07	***	***	***
13 BO	0.90	1.30	0.95	0.95	0.00	0.70	1.10	0.80	0.90	0.75	1.00	0.90	0.07	***	***	***
14 BO	1.10	1.10	1.20	1.30	1.60	1.20	1.45	1.10	0.70	0.75	0.90	1.00	0.08	***	ns	**
17 BO	1.00	0.90	1.25	1.10	0.70	0.90	0.90	0.70	0.70	1.00	0.90	0.85	0.08	**	**	ns
20 BO	0.00	0.75	0.85	1.05	0.00	1.10	1.35	1.10	0.00	0.00	0.00	0.85	0.05	***	***	***
24 BO	0.85	0.00	0.00	1.20	0.75	0.00	1.10	1.10	0.00	0.00	0.00	0.75	0.05	***	***	***

[a] EO activity is indicated by the width of the inhibition zone (cm) around the paper disc; [b] A, December 2012; B, January 2013; C, February 2013; D, April 2013. Results indicate mean values of four replicates (carried out in duplicate and repeated twice).
Abbreviations: SEM, standard error of means; CV, cultivar; CT, collection time. P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns = not significant.

Table 1.2. Inhibitory activity[a] of lemon EOs extracted from fruits harvested at different collection times[b] against *Staphylococcus aureus.*

Strain	*Citrus limon* L. Burm. spp. cultivars												SEM	Statistical significance[a]		
	Femminello Santa Teresa				Monachello				Femminello Continella							
	A	B	C	D	A	B	C	D	A	B	C	D		CV	CT	CV*CT
C1/5634-MSSA	1.05	0.75	1.15	0.90	0.90	0.80	1.35	1.15	1.00	0.85	0.00	1.10	1.15	***	***	***
C4/6561,1-MSSA	1.10	1.15	0.95	1.30	1.30	1.30	1.30	1.00	1.10	0.75	0.70	1.10	0.06	***	***	***
C38/249,1-MSSA	1.10	1.20	1.55	1.20	1.10	0.95	1.50	0.80	0.70	0.00	0.00	0.75	0.06	***	***	***
C45/12425-MSSA	1.10	1.35	1.15	0.80	1.10	1.50	1.10	1.30	0.85	0.00	0.00	0.85	0.07	***	**	***
195-MRSA	0.95	0.00	1.00	0.90	1.00	0.90	1.15	1.50	0.90	0.00	0.70	0.70	0.07	***	***	***
1313-MRSA	1.00	0.70	1.05	0.90	1.10	0.90	1.30	1.40	0.85	0.00	0.85	0.85	0.07	***	***	**
581-MRSA	0.90	0.85	1.05	1.15	0.95	0.80	1.30	1.10	0.80	0.80	0.85	0.00	0.06	***	***	***
340-MRSA	1.00	0.90	1.70	1.45	0.90	0.90	1.60	1.10	0.70	0.75	0.00	1.15	0.07	***	***	***
4ADI MRSA	1.00	0.85	1.65	1.20	0.70	0.75	1.15	1.20	0.90	0.70	0.85	0.00	0.06	***	***	***
7ADI MSSA	0.70	0.70	1.10	1.40	0.85	0.80	1.20	1.10	0.85	0.90	0.00	0.80	0.07	***	***	***
14LU MRSA	1.00	1.00	0.90	1.15	1.00	0.90	1.00	1.45	0.85	0.75	0.75	0.85	0.07	***	*	ns
16 MSSA	1.10	0.70	1.45	0.95	0.90	0.75	1.15	1.25	0.85	0.00	0.70	1.30	0.07	***	***	***
20 ADI MRSA	1.00	0.75	1.40	1.10	0.90	0.85	1.50	0.70	0.75	0.00	0.75	0.80	0.06	***	***	***
21 ADI MRSA	0.90	0.75	0.85	0.95	0.90	0.90	1.45	1.00	0.75	0.75	0.90	0.85	0.07	***	**	**
62 MRSA	0.95	1.10	1.40	1.15	0.85	0.90	1.15	1.00	0.85	0.90	0.00	0.00	0.07	***	**	***
68 MRSA	0.80	0.95	1.25	1.10	0.75	0.95	1.55	1.55	0.70	0.75	0.00	1.90	0.07	***	***	***
106 MRSA	0.85	0.95	1.10	1.05	0.80	1.00	1.50	0.70	0.70	0.00	0.70	1.05	0.08	***	***	***
109 MRSA	0.80	0.85	1.10	0.90	1.05	0.70	1.50	1.05	0.65	0.00	0.80	0.90	0.07	***	***	***
156 MRSA	0.95	0.85	1.20	1.50	1.00	0.90	1.35	1.60	0.70	0.90	0.85	1.00	0.06	***	***	ns
168 MRSA	0.90	0.90	0.90	1.15	0.95	0.85	1.10	1.15	0.85	0.00	0.90	0.90	0.07	***	***	***
473 MRSA	0.85	0.90	1.30	0.00	1.10	0.85	1.50	0.00	0.85	0.75	1.05	1.00	0.07	*	***	***
493 MRSA	0.90	0.95	1.50	1.05	0.80	0.95	1.50	1.10	0.90	0.80	0.85	0.90	0.08	**	***	**
637 MRSA	0.95	0.90	1.00	1.40	0.75	1.05	1.00	1.50	0.75	0.70	0.00	2.40	0.06	*	***	***
734 MSSA	0.90	0.80	1.40	1.20	0.85	0.70	1.30	0.95	0.85	0.75	0.00	1.15	0.06	***	***	***
750 MSSA	1.05	0.85	0.90	1.35	0.75	0.85	1.60	1.25	0.90	0.80	0.80	1.40	0.05	*	***	***
E36GI MRSA	1.00	0.70	0.95	1.70	0.70	0.70	1.00	1.40	0.80	0.90	0.80	1.45	0.07	ns	***	*

[a] EO activity is indicated by the width of the inhibition zone (cm) around the paper disc; [b] A, December 2012; B, January 2013; C, February 2013; D, April 2013. Results indicate mean values of four replicates (carried out in duplicate and repeated twice).

Abbreviations: SEM, standard error of means; CV, cultivar; CT, collection time. P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns = not significant.

Table 1.3. Inhibitory activity[a] of lemon EOs extracted from fruits harvested at different collection times[b] against *Salmonella enterica.*

Strain	*Citrus limon* L. Burm. spp. cultivars												SEM	Statistical significance[a]		
	Femminello Santa Teresa				Monachello				Femminello Continella							
	A	B	C	D	A	B	C	D	A	B	C	D		CV	CT	CV*CT
S. Abony 50398	0.00	1.50	0.00	0.00	0.00	1.30	0.00	0.00	0.00	1.10	0.00	0.00	0.05	*	***	*
S. Agona 50360	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	***	***	***
S. Blockley 50314	1.10	0.00	1.10	0.00	0.00	1.10	0.00	0.00	0.00	0.00	0.00	0.00	0.05	***	***	***
S. Bredeney 50374	1.15	1.50	0.00	0.00	0.00	1.30	0.00	0.00	0.00	0.00	0.00	0.00	0.04	***	***	***
S. Derby 50399	0.95	1.10	0.00	1.50	0.00	1.50	0.00	1.10	0.00	0.00	0.00	1.70	0.07	***	***	***
S. Enteritidis 50339	0.90	1.00	1.40	1.60	1.30	1.40	1.50	1.50	0.00	1.10	0.00	1.30	0.07	***	***	***
S. Hadar 50272	1.10	1.10	1.45	1.50	1.20	1.30	1.55	1.50	1.10	0.00	1.10	1.10	0.08	***	***	***
S. Infantis 50270	0.70	0.00	0.00	1.45	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.50	0.04	***	***	***
S. Muenchen 50393	0.70	0.00	0.00	1.10	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.05	***	***	***
S. Napoli 50376	0.90	0.00	1.30	1.30	0.00	1.30	0.00	0.80	0.00	0.00	0.00	0.00	0.15	***	*	***
S. Newport 50404	0.90	1.30	1.30	1.10	0.00	1.30	1.30	1.10	0.00	0.00	1.30	1.10	0.09	***	***	***
S. Panama 50347	0.00	0.00	0.00	1.10	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.04	***	***	***
S. Saintpaul 50415	0.00	0.00	0.00	0.00	0.00	0.00	1.35	0.00	0.00	0.00	0.00	0.00	0.04	***	***	***
S. Thompson 50280	0.00	0.90	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.90	0.00	0.00	0.05	***	***	***

[a] EO activity is indicated by the width of the inhibition zone (cm) around the paper disc; [b] A, December 2012; B, January 2013; C, February 2013; D, April 2013. Results indicate mean values of four replicates (carried out in duplicate and repeated twice).
Abbreviations: SEM, standard error of means; CV, cultivar; CT, collection time. P value: *, $P \leq 0.05$; ***, $P \leq 0.001$.

Table 1.4. Inhibitory activity[a] of lemon EOs extracted from fruits harvested at different collection times[b] against *Enterobacter* spp..

Strain	*Citrus limon* L. Burm. spp. cultivars												SEM	Statistical significance[a]		
	Femminello Santa Teresa				Monachello				Femminello Continella							
	A	B	C	D	A	B	C	D	A	B	C	D		CV	CT	CV*CT
1435 UTIN	1.30	0.00	1.40	1.85	1.50	1.25	1.05	1.70	1.10	0.00	0.95	0.90	0.06	***	***	***
4 UTIN	1.40	1.45	1.45	1.05	1.40	1.10	1.50	1.15	0.00	1.05	0.90	1.00	0.07	***	***	***
5UTIN	1.55	1.35	1.30	1.60	1.25	1.35	1.35	1.65	1.00	1.15	1.00	1.30	0.06	***	***	*
7UTIN	1.25	0.00	0.00	1.65	1.10	0.00	0.00	1.65	1.20	0.00	0.00	0.85	0.04	***	***	***
8UTIN	0.00	1.10	1.20	1.50	0.00	1.20	1.30	1.25	0.00	1.10	1.20	1.45	0.06	ns	***	ns
9UTIN	0.90	1.65	1.25	1.50	0.80	1.50	1.10	1.10	0.70	0.90	0.90	1.35	0.09	***	***	**
10UTIN	0.80	1.40	1.00	1.20	1.00	1.55	1.20	1.40	0.80	1.10	1.15	1.30	0.05	***	***	**
12UTIN	1.30	1.50	1.45	1.40	0.70	1.65	1.50	1.90	0.00	1.45	1.25	1.15	0.07	***	***	***
17UTIN	0.00	1.30	0.00	1.40	0.00	1.60	0.90	1.90	0.00	1.25	1.05	1.20	0.05	***	***	***
19UTIN	0.95	1.30	0.00	1.35	1.00	1.20	0.00	1.60	1.05	1.25	0.00	0.95	0.04	**	***	***
20UTIN	1.60	1.15	0.80	1.00	1.50	1.45	0.80	0.00	1.50	1.00	0.00	0.00	0.04	***	***	***
28UTIN	1.55	1.45	0.80	1.50	0.90	1.45	0.80	1.90	0.00	1.30	0.80	1.00	0.06	***	***	***
29UTIN	0.90	1.30	0.80	1.50	1.10	1.30	0.80	1.90	0.90	1.45	0.80	1.25	0.08	*	***	**
30UTIN	1.10	0.95	0.80	1.50	1.45	1.25	0.80	1.00	0.00	0.95	0.80	1.05	0.05	***	***	***
31UTIN	1.40	1.40	0.80	1.60	1.80	1.40	0.80	1.25	0.00	1.50	0.80	1.05	0.05	***	***	***
33UTIN	1.90	1.20	0.80	1.25	1.40	1.40	0.80	1.45	0.90	0.95	0.80	1.30	0.06	***	***	***
35UTIN	0.00	0.00	0.00	0.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	***	***	***
36UTIN	0.95	0.00	0.90	1.60	1.10	0.00	1.05	1.35	0.95	0.00	1.00	1.15	0.05	*	***	**
E. hormaechei 1	1.55	1.65	1.45	1.60	1.60	1.45	1.10	1.45	1.45	1.40	1.00	1.50	0.06	***	***	ns
E. hormaechei 2	1.45	0.90	1.40	1.90	1.15	1.30	1.10	1.50	0.00	1.10	1.00	1.65	0.08	***	***	***
E. hormaechei 6	1.45	0.95	1.00	1.40	1.55	1.20	1.70	1.30	1.20	0.95	1.10	1.50	0.06	***	***	***
E. hormaechei 7	1.40	0.95	1.65	1.40	1.10	1.10	1.35	1.40	0.00	1.10	0.95	1.20	0.06	***	***	***
E. hormaechei 8	1.70	1.10	1.70	1.35	1.90	0.70	1.40	1.80	0.80	0.95	1.50	1.10	0.08	***	***	***
E. hormaechei 11	1.65	1.50	1.55	2.10	1.90	1.10	1.30	1.85	1.55	1.40	1.00	1.30	0.08	***	***	***
E. hormaechei 13	1.60	0.90	1.50	1.50	1.60	1.30	1.70	1.25	1.60	1.00	1.05	1.90	0.09	ns	***	***
E. hormaechei 19	1.60	1.40	1.50	1.35	1.40	1.30	1.60	1.65	1.15	1.00	1.50	1.70	0.08	*	***	**
E. hormaechei 20	1.45	1.10	1.10	1.50	1.80	1.25	1.10	1.15	0.00	0.00	1.10	0.00	0.07	***	***	***
E. hormaechei 31	1.35	1.30	1.45	1.40	1.40	1.25	1.10	1.45	1.30	0.90	1.10	1.45	0.08	*	**	*
E. cloacae 24	1.50	1.15	1.50	1.20	1.25	1.30	1.50	1.45	0.70	1.30	1.05	1.10	0.08	***	ns	**
E. cloacae 25	1.50	1.40	1.55	1.45	1.40	1.45	2.10	0.90	1.20	1.15	1.50	0.00	0.06	***	***	***
E. cloacae 13A	1.05	1.20	1.45	1.70	0.70	1.15	1.40	1.90	1.30	1.20	0.00	1.60	0.06	***	***	***
E. cloacae 62A	1.00	1.30	1.20	1.30	1.05	1.10	1.30	1.40	0.00	1.05	1.30	1.30	0.07	***	***	***
E. cloacae 32A	1.00	1.10	1.50	1.25	1.50	1.40	1.50	1.50	1.25	1.40	1.00	0.80	0.07	***	ns	***
E. cloacae 43B1	11.00	1.65	1.45	1.70	1.15	1.60	1.15	1.45	1.00	1.70	1.35	1.10	0.07	**	***	**
E. sakazaki 2B	2.65	0.90	0.80	0.80	2.55	1.40	0.80	1.20	1.50	1.20	1.20	0.90	0.05	***	***	***
E. sakazaki 23A	1.80	0.70	0.80	0.80	1.45	1.40	0.80	1.20	1.20	1.15	1.20	0.90	0.05	***	***	***
E. amnigenus 70B3	1.05	1.45	0.80	1.70	0.95	1.30	0.80	1.25	0.00	1.10	1.20	0.95	0.06	***	***	***
E. amnigenus 60A2	0.80	1.10	1.50	0.90	0.90	1.05	1.20	1.25	0.00	0.85	1.05	0.00	0.06	***	***	***

[a] EO activity is indicated by the width of the inhibition zone (cm) around the paper disc; [b] A, December 2012; B, January 2013; C, February 2013; D, April 2013. Results indicate mean values of four replicates (carried out in duplicate and repeated twice).
Abbreviations: SEM, standard error of means; CV, cultivar; CT, collection time. P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns = not significant.

1.2.2. Chemical composition of lemon EOs

In order to determine the seasonal and cultivar variability of EOs, the chemical composition was analyzed by GC/MS (Table 1.5). A total of 42 chemicals were clearly identified among the three cultivars at four collection times. Three main classes of compounds were found: monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. In general, EOs contain about 20–60 components at different concentrations (Bakkali *et al.*, 2008).

The monoterpene hydrocarbon fraction dominated all EOs, while sesquiterpene hydrocarbons constituted the quantitative percentage less relevant. Camphene was only detected in February EO of the cultivar Femminello Continella, 1-nonanol was not found for any collection time of Monachello, while β-citronellale was not identified in EOs of Femminello Santa Teresa and Femminello Continella. The chemicals quantitatively dominant in all EOs were D-limonene, γ-Terpinene and β-pinene among the monoterpene hydrocarbons, whereas trans-geraniol, cis-geraniol, 4-terpineol and α-citral were the oxygenated monoterpens found at consistent levels. The compound quantitatively dominant in all EOs was D-limonene, which is reported to represent until the 70% of citrus EOs (Bakkali *et al.*, 2008).

No statistical differences ($P>0.05$) were found regarding EO and ripening stage for camphene, fenchol, borneol, α–citronellol, β-citronellale, 2-octen-1-ol,3,7-dimethyl and bicyclo[3.1.1]hept-2-ene,2,6,6-trimethyl. α–phellandrene and 1-nonanol were not significantly different among seasons ($P>0.05$), while β-ocimene, β-citronellol, β-farnesene and cis-α-bisabolene were not significantly different among EOs ($P>0.05$). All other compounds resulted quantitatively different among samples and collection

times ($P \leq 0.05$ – $P \leq 0.001$) and these differences may be responsible for the diverse sensitivity of the strains to the EOs. Other studies evidenced seasonal variations in the amounts of many components in lemon EOs (Staroscik *et al.*, 1982; Vekiari *et al.*, 2002) and also in other citrus than lemon EOs (Frizzo *et al.*, 2004). However, our results did not agree with the previous studies for some main components of EOs which, in our study, were more concentrated in later collection times.

The comparison of the chemical composition of the EOs analysed suggested that the compounds mainly implicated in the process of bacterial inhibition could be the oxygenated monoterpenes. Our results confirmed previous observations regarding this class of chemicals, especially phenolic substances, that exhibits a stronger antimicrobial activity than monoterpenes hydrocarbon (Knobloch *et al.*, 1986; Soković *et al.* 2002; Soković *et al.*, 2006). The last hydrocarbon compounds are characterised by a low water solubility which limits their diffusion through the medium. Furthermore, their inactivity is closely related to their limited hydrogen bound capacity (Griffin *et al.*, 2000). The oxygenated monoterpenes have been found at lower amounts than hydrocarbon monoterpenes. Burt (2004) stated that the minor components of EOs are more effective in the inhibitory process than the compounds present at higher concentrations.

Table 1.5. Chemical composition of lemon EOs (LSM) at four[a] different seasonal harvesting times.

Compound	LRI[b]	Femminello Santa Teresa				Monachello				Femminello Continella				SEM	Statistical significance[c]		
		A	B	C	D	A	B	C	D	A	B	C	D		EO	Season	EO x Season
Monoterpene hydrocarbons		63.28	80.87	83.61	78.84	67.46	82.69	79.17	82.96	76.74	86.89	88.83	85.41				
α-Thujene	930	0.16	0.24	0.27	0.20	0.21	0.37	0.25	0.28	0.19	0.30	0.25	0.19	0.03	*	**	ns
α-Pinene	939	0.62	1.10	1.16	0.92	0.95	1.57	1.02	1.22	1.01	1.29	1.17	0.96	0.06	***	***	**
Camphene	954	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	ns	ns	ns
Sabinene	976	0.39	0.69	0.82	0.82	0.86	1.04	0.99	1.21	0.22	0.83	0.66	0.87	0.07	***	***	ns
β-Pinene	981	4.86	5.65	7.09	7.64	8.64	12.28	8.72	10.36	6.98	8.86	8.50	8.04	0.34	***	***	***
β-Myrcene	990	0.94	1.52	1.60	1.30	0.89	1.28	1.29	1.32	1.24	1.44	1.53	1.29	0.06	**	***	*
α-Phellandrene	1007	0.00	0.00	0.01	0.00	0.02	0.04	0.02	0.00	0.04	0.05	0.06	0.02	0.01	**	ns	ns
(+)-4-Carene	1020	0.25	0.26	0.32	0.31	0.31	1.32	0.41	0.36	0.29	0.41	0.45	0.35	0.02	***	***	***
p-Cymene	1026	0.14	0.07	0.05	0.18	0.28	0.27	0.21	0.20	0.16	0.04	0.00	0.05	0.02	***	***	**
D-Limonene	1029	48.94	62.46	61.71	56.66	46.50	51.14	52.81	55.08	57.93	62.46	63.33	61.72	2.20	***	**	ns
β-Ocimene	1035	0.09	0.11	0.17	0.18	0.11	0.12	0.17	0.28	0.06	0.17	0.30	0.23	0.04	ns	**	ns
gamma-Terpinene	1014	6.47	8.31	9.83	10.03	8.66	13.22	12.51	11.97	8.17	10.42	11.81	10.95	0.40	***	***	*
(+)-2-Caren	1002	0.39	0.42	0.53	0.55	0.00	0.00	0.69	0.62	0.42	0.58	0.70	0.68	0.02	***	***	***
Oxygenated monoterpenes		10.49	12.9	13.34	13.36	8.58	14.59	17.3	12.59	4.56	6.64	7.82	7.59	0.7			
Octanal	1006	0.00	0.00	0.09	0.09	0.15	0.03	0.11	0.07	0.00	0.00	0.00	0.11	0.01	***	***	***
1-Octanol	1090	0.00	0.04	0.09	0.13	0.03	0.14	0.09	0.11	0.00	0.06	0.08	0.03	0.02	**	***	*
Linalol	1098	0.87	0.55	0.65	0.58	0.52	0.91	0.79	0.47	0.43	0.53	0.62	0.33	0.02	***	***	***
Nonanal	1015	0.08	0.10	0.14	0.20	0.16	0.19	0.21	0.18	0.09	0.11	0.10	0.21	0.01	***	***	***
Fenchol	1112	0.04	0.02	0.03	0.03	0.03	0.06	0.03	0.01	0.01	0.02	0.04	0.07	0.01	ns	ns	***
(R)-(+)-Citronellal	1145	0.05	0.07	0.07	0.07	0.07	0.14	0.15	0.09	0.04	0.03	0.02	0.05	0.00	***	***	***
Borneol	1172	0.06	0.07	0.08	0.00	0.08	0.07	0.10	0.00	0.00	0.00	0.00	0.10	0.03	ns	ns	*
1-Nonanol	1155	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.02	0.11	0.13	0.00	0.02	***	ns	**
4-Terpineol	1177	1.21	0.77	1.09	1.34	1.26	2.19	1.69	1.28	0.77	1.29	1.51	1.41	0.05	***	***	***
α-Terpineol	1188	1.86	0.98	1.39	1.68	1.41	2.39	1.86	1.53	0.79	1.31	1.72	2.34	0.05	***	***	***
Decanal	1209	0.03	0.04	0.06	0.08	0.06	0.08	0.08	0.08	0.03	0.03	0.02	0.09	0.01	***	***	***
cis-Geraniol	1255	1.31	1.30	1.50	1.36	0.47	1.37	1.61	1.35	0.33	0.84	1.07	0.23	0.08	***	***	***
β-Citronellol	1226	0.16	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.00	0.12	0.11	0.00	0.07	ns	*	*
α-Citronellol	1232	0.00	0.25	0.19	0.25	0.00	0.00	0.22	0.31	0.00	0.00	0.00	0.03	0.11	ns	ns	ns

Table 1.5. Continue.

β-Citral	1337	1.09	2.30	1.91	1.72	1.21	1.23	2.66	1.69	0.43	0.06	0.02	0.59	0.04	***	***	***					
trans-Geraniol	1254	2.13	2.03	2.08	1.99	0.57	2.45	2.41	1.82	0.44	1.28	1.53	0.32	0.06	***	***	***					
α-Citral	1358	0.85	2.89	2.37	2.23	1.53	1.42	3.35	2.12	0.55	0.08	0.03	0.78	0.05	***	***	***					
β-Citronellale	1314	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.02	ns	ns	ns					
Geranyl acetate	1383	0.29	0.76	0.87	0.82	0.39	0.52	0.88	0.74	0.19	0.24	0.35	0.41	0.02	***	***	***					
Neryl acetate	1365	0.46	0.73	0.73	0.73	0.64	0.87	1.06	0.74	0.44	0.53	0.47	0.49	0.02	***	***	***					
Sesquiterpene hydrocarbons		0.52	1.18	1.27	1.33	0.76	0.97	1.56	1.5	0.64	0.76	1.28	1.49	0.05								
Caryophyllene	1419	0.09	0.17	0.16	0.17	0.15	0.17	0.24	0.21	0.11	0.11	0.15	0.16	0.01	***	***	**					
α-Bergamotene	1493	0.16	0.35	0.40	0.42	0.22	0.28	0.47	0.46	0.19	0.23	0.40	0.48	0.01	**	***	***					
β-Farnesene	1450	0.00	0.03	0.03	0.03	0.02	0.02	0.03	0.03	0.00	0.02	0.03	0.03	0.00	ns	***	**					
cis-α-Bisabolene	1508	0.02	0.07	0.05	0.05	0.03	0.02	0.06	0.05	0.03	0.03	0.05	0.06	0.01	ns	**	ns					
β-Bisabolene	1502	0.25	0.56	0.63	0.66	0.34	0.48	0.76	0.75	0.31	0.37	0.65	0.76	0.02	***	***	***					
Others		0.21	0.17	0.03	0.04	0.05	0.41	0.06	0	0.03	0.19	0.04	0.02	0.19								
Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl		0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.10	ns	ns	ns					
2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, trans		0.04	0.02	0.03	0.04	0.05	0.06	0.06	0.00	0.03	0.04	0.04	0.02	0.01	*	***	***					
2-Octen-1-ol, 3,7-dimethyl		0.17	0.15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0.15	0,00	0,00	0.08	ns	ns	ns					

[a] A, December 2012; B, January 2013; C, February 2013; D, April 2013. [b] Linear retention index on SLB-5MS column. [c] P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001; ns = not significant. Abbreviations: LSM, least square means; SEM, standard error of means.

1.3. Conclusions

The ecological role of EOs is mainly to protect the plants, other than interact with other organisms. Due to their antimicrobial properties, they could find several applications as alternatives to synthetic chemical products to pursue biopreservation objectives (Settanni *et al.*, 2012).

Citrus essential oils have been object of many studies because of the economic importance of their production. Moreover, the antibacterial properties of citrus EOs have been long recognized, but the recent interest in alternative naturally derived antimicrobials, requested not only by consumers but also by legal authorities, has determined a "renewal of interest" of their application in the preservation of foods.

In this study, the inhibitory spectra of the EOs extracted from the peel of the fruits of three cultivars of *Citrus limon* L. Burm. was affected significantly by the time of collection. However, the inhibitory power of EOs was found to be strain-dependent. In general, all EOs tested were more effective against the Gram-positive rather than Gram-negative bacteria.

Chemical analyses revealed that monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons were the main components of all EOs. Forty-two compounds were identified and their quantitative differences among samples and collection times may be responsible for the diverse sensitivity of the strains to the EOs.

Chapter 2

Antilisterial effect of Citrus essential oils and their performance in edible film formulations

2.1. Materials and Methods

2.1.1. *Listeria monocytogenes* strains

Seventy-six strains of *L. monocytogenes* were used in this study. All strains, belonging to the Department of Biotechnology – Microbiology Area, ETSIAMN (Universitat Politecnica de Valencia, Spain), were previously isolated from food matrices including dairy products, fish, meat and vegetables, following the ISO method 11290–1:1996 (ISO 11290-1:1996). Bacterial strains were stored in cryovials (Microbank™ Prolab Diagnostics, Austin, USA) at –80°C. The strains were reactivated and sub-cultured onto Tryptic Soy Agar (TSA, Merck Millipore, Darmstadt, Germany) incubated overnight at 37°C.

2.1.2. Citrus samples and extraction of EOs

The EOs analyzed in this study were obtained from the peels of eight different citrus fruits cultivated in Sicily (Table 2.1) and collected during March 2014.

Samples EO M1 and EO L2 derived from mature trees cultivated in the collection orchard "Parco d'Orleans" of the Agricultural Faculty of Palermo, while samples EO O3, EO O4, EO O5, EO O6, EO M7 and EO L8 from the "Azienda Sperimentale Palazzelli C.R.A. e Centro di ricerca per l'agrumicoltura e le colture mediterranee Contrada Palazzelli Scordia" (CT, Italy).

After peeling, the peels were immediately subjected to hydrodistillation for 3 h using a Clevenger-type apparatus (Comande, Palermo, Italy) collecting the oil in hexane. EOs were dried over anhydrous sodium sulfate and stored at 4°C in air-tight sealed glass vials covered with aluminum foil.

Table 2.1. Sicilian EOs used in the antilisterial screening.

EO	Species	Variety	Sperimental Orchard
M1	Mandarin (*Citrus reticulata* Blanco)	Mandarino Tardivo di Ciaculli	Campo dei Tigli (Palermo)
L2	Lemon (*Citrus limon* L. Burm.)	Femminello Santa Teresa	Campo dei Tigli (Palermo)
O3	Sweet Orange (*Citrus sinensis* L. Osbeck)	Moro Nucellare	Campo Palazzelli (Acireale)
O4	Sweet Orange (*Citrus sinensis* L. Osbeck)	Lane Late	Campo Palazzelli (Acireale)
O5	Sweet Orange (*Citrus sinensis* L. Osbeck)	Tarocco Tardivo	Campo Palazzelli (Acireale)
O6	Sweet Orange (*Citrus sinensis* L. Osbeck)	Sanguinello Nucellare	Campo Palazzelli (Acireale)
M7	Hybrid (Horoval clementine x Tarocco orange)	Alkantara mandarin ®	Campo Palazzelli (Acireale)
L8	Lemon (*Citrus limon* L. Burm.)	Limone KR (Siracusano)	Campo Palazzelli (Acireale)

2.1.3. Chemical characterization

GC/MS analysis of the EOs was performed by gas chromatography couple with mass spectrometry (GC/MS) (EI) on a GCMSQP2010 (Shimadzu, Milan, Italy). NIST 21,107,147 library was used for data acquisition. The analysis was carried out through a fused silica capillary column SLB-5MS (5% diphenyl:95% methylsiloxane) 30 m x 0.25 i.d. x 0.25 mm film thickness (Supelco, Milan, Italy); helium gas was used as the carrier gas at a constant linear rate 30 cm s^{-1} (30.6 kPa); split/splitless injector port; injector temperature 250°C; injection mode split (split ratio 100:1). The oven temperature was programmed as follows: 50°C, hold 3 min; 3°C/ min to 240°C; 15°C/min to 280°C, hold 1 min. MS scan conditions were: source temperature 200°C, interface temperature 250°C, EI energy 70 eV; mass scan range 40e400 amu. GC/MS analysis was carried out in duplicate.

2.1.4. Screening of antilisterial activity

The antibacterial activity of the eight EOs against *L. monocytogenes* strains was tested by the paper disc diffusion method applied by Kelmanson *et al.* (2000) and with the modifications of Militello *et al.* (2011). Bacterial cells were grown at 37°C overnight before tests on tryptone soy broth (TSB). A concentration of about 10^7 CFU/ml of each strain was inoculated into 7 ml of TSA soft agar (0.7%, w/v) and poured onto TSA. Sterile filter paper discs (Filter-Lab Anoia, Spain) of 6 mm diameter were placed onto the surface of the double agar layer and soaked with 10 ml of each undiluted EO. Sterile water was used as negative control. Antibacterial activity was positive when a definite halo of inhibition (in cm) was detected around the paper disc. Each test was performed in duplicate and the experiments were repeated twice. Resulting data were subjected to statistical analysis using the ANOVA procedure with Statistica 10 (Statsoft, USA) software. Differences between means were determined by Tukey's multiplerange test.

2.1.5. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was used to measure the antibacterial activity, since it represents a common method to express the EO antibacterial performances (Burt, 2004). MIC is defined as the lowest concentration of an active compound inhibiting visible growth of the tested organisms (Karapinar *et al.*, 1987). The strength of the antibacterial activity is determined using dilutions of EO in order to determine the end-point by means of the disc diffusion assay. Each *L. monocytogenes* strain was inoculated into TSA at 10^6 CFU/ml, the paper discs were

disposed onto the agar surface, soaked with 10 ml of the serial dilutions of EOs and incubated O/N at 37°C. Serial dilutions (dilution factor = 2) were obtained with dimethyl sulfoxide (DMSO, SigmaeAldrich, Milan, Italy). DMSO alone was used as negative control. Each test was performed in duplicate and the experiments were repeated twice.

2.1.6. Viability of *L. monocytogenes* strains by fluorescence microscopy

The viability of the most sensitive *L. monocytogenes* strains after treatment with EOs was evaluated by Viability Kit LIVE/DEAD® BacLight™(Molecular Probes Inc. Eugene Oregon) and plate counts onto TSA. The viability test was carried out with the strains inoculated at a final density of 10^4 CFU/ml in broth containing 0.125% (v/v) EO. Cells were counted as follows: 500 ml of each broth collected at 0, 1, 2, 4 and 6 h of treatment with EO was added with 0.8 ml of the fluorochromes mix (1:1 v/v, EO/mix) and incubated in darkness at room temperature for 15 min. Five microliters of the resulting mixture were placed onto a poly-L-lisina slide (Poly-Prep® slides, Sigma Diagnostics, U.S.A.). After 10 min of incubation at room temperature, the counts were carried out by the epifluorescence microscope Olympus BX 50 (with a mercury bulb of 100W) equipped with a double filter (XF 53, Omega) (Olympus Optial Co., Hamburg, Germany). Digital colored photos were taken with Olympus DP10 digital camera (Figure 2.2).

2.1.7. Antilisterial effect of edible EOs-based films

Chitosan-based (CH) and methylcellulose-based (MC) films were used to perform the antilisterial assay (Figure 2.1). High molecular weight chitosan (1.2 Pa s viscosity at 1% w/w in 1% w/w glacial acetic acid, acetylation degree: 4.2%, SigmaeAldrich, USA) was dispersed at 1% w/w in an aqueous solution of acetic acid (1% v/w) and stirred overnight at room temperature. Methylcellulose (0.3–5.6 Pa s viscosity at 1% w/w in water solution, VWR BDH ProLabo, Spain) was dispersed in distilled water (1% w/w) and heated up to 80°C to promote solubilization. Once the polymer solutions were obtained, each EO was added at a concentration of 0.5% (polymer: EO ratio 2:1) and stirred for 10 min. The mixtures were then sonicated by the Vibra Cell VCX750 sonicator (Sonics & Materials, Inc., USA) at 20 kHz and 40% power for 480 s (1 s on and 1 s off) in order to obtain the film forming dispersions (FFD). FFDs were casted in plates (diameter 53 mm), weighted up to 6.7 g, to keep polymer amount constant in dry films (30 g polymer/m2). The films were dried at room temperature and 60% relative humidity (RH).

The surface of TSA plates (10 g) was seeded with 0.35 ml of cell suspensions (10^4 CFU/ml) and covered with CH and MC films. Inoculated coated TSA and inoculated non-coated TSA dishes were used as controls. Plates were then sealed with parafilm to avoid dehydration and incubated at 37°C for 0, 8 and 24 h and at 8°C for 0,1, 3 and 7 d. The two temperatures were chosen to investigate the effect of the EOs at the optimal growth temperature for the test strains (37°C) and simulating the conditions of a domestic refrigerator (8°C).

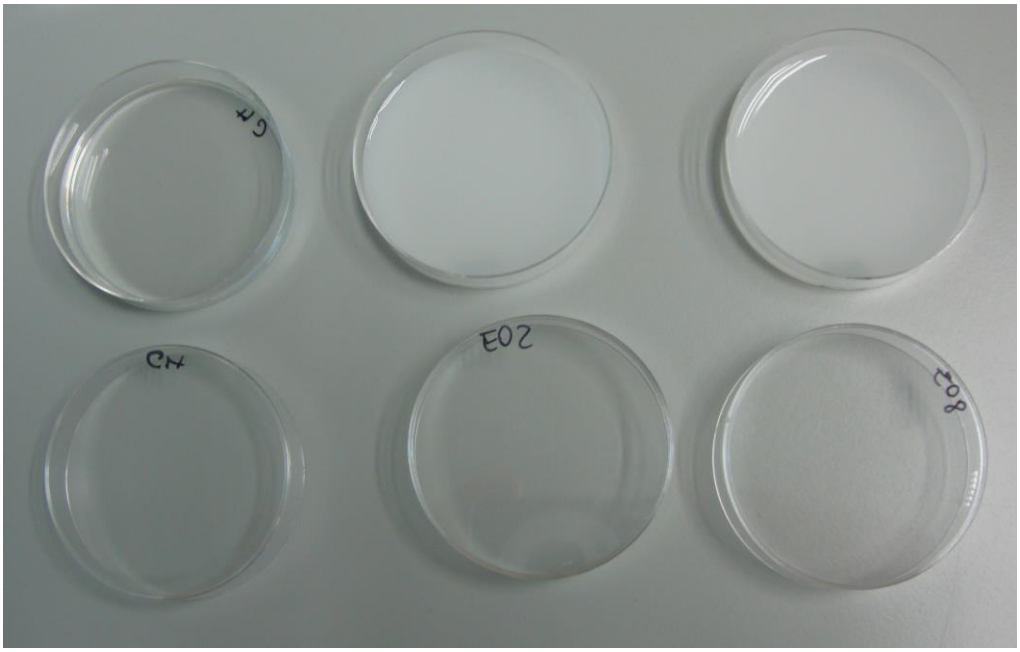


Figure 2.1. Casted FFDs with and without experimental EOs.

The agar layer was then aseptically removed from each Petri dish and placed into a sterile stomacher bag with 90 ml of Peptone Water (Merck Millipore, Darmstadt, Germany) and homogenized for 60 s in the stomacher Bag Mixer 400 (Interscience, Saint Nom, France).

Serial dilutions were set up with Ringer's solution (SigmaeAldrich, Milan, Italy) and 0.1 ml of cell suspensions were spread plated onto TSA plates. Colonies were enumerated after 24 h at 37°C. The experiment was carried in duplicate.

2.1.8. Microstructure

Film microstructure was observed by Scanning Electron Microscopy in cross-sectioned cryofractured specimens, using a JEOL JSM-5410 (Tokyo, Japan) electron microscope in order to qualitatively assess the EOs incorporation into the polymeric

matrix (Figure 2.3). The films (3 samples per formulation) were equilibrated in P_2O_5 to eliminate water prior cryofracturing them by immersion in liquid nitrogen. Afterwards, cryo-fractured samples were mounted on copper stubs. After gold coating, the images were captured using an accelerating voltage of 10 kV.

2.2. Results and discussion

2.2.1. Screening of the antilisterial activity

The results of the disc diffusion assay are shown in Table 2.2. All EOs resulted statistically different ($P < 0.001$) in inhibiting the strains tested, confirming previous statements that the sensitivity to natural antimicrobial compounds is strain-dependent (Settanni et al., 2014). EO L2 and EO L8 showed the widest spectra of inhibitory activity.

In particular, EO L2 inhibited all tested strains and for thirty-five of them the clear halos were larger than 10 mm. Except *L. monocytogenes* LM68, all other strains were sensitive to EO L8 and the halos were registered at diameters larger than 10 mm for twenty-nine indicator strains.

Regarding the inhibition by the other EOs, only *L. monocytogenes* LM10, LM16, LM35 and LM69 were particularly sensitive. On the contrary, strains LM09, LM29, LM63, LM66, LM68 were not inhibited by at least three EOs. EOs O3 and M7 did not show interesting antilisterial activities.

Table 2.2. Inhibitory activity[a] of citrus EOs against *Listeria monocytogenes* isolated from food tested by disc diffusion assay.

Strain code	EO M1	EO L2	EO O3	EO O4	EO O5	EO O6	EO M7	EO L8	Statistical significance[b]	Source of isolation[c]
LM01	0.6	0.8	0.6	0.6	0.6	0.6	0.7	1	***	M
LM02	0.7	0.8	0	0.6	1	0.8	0.8	1	***	M
LM03	0.8	0.8	0	0	0.8	1	0.6	0.8	**	M
LM04	1	1	0.6	0.6	0.6	0.6	0.6	0.6	***	D
LM05	0	0.8	0	0.8	0.6	1	0.6	1.4	**	D
LM06	0.7	0.8	0.6	0.6	0.6	0.8	0.7	0.8	***	M
LM07	0.6	0.8	0.7	0.6	0.6	0.6	0.7	0.8	***	D
LM08	0.6	0.6	0.8	0.6	0.7	0.8	0.6	1.1	***	F
LM09	0.6	0.8	0	0.8	0	0.8	0	0.8	*	F
LM10	0.8	0.8	1	0.8	1.4	1	1	1	***	D
LM11	0.6	1	0.6	0	0.8	0.8	0.8	0.8	***	F
LM12	0.8	0.9	0.6	0.6	0.6	0.8	0.6	0.8	***	M
LM13	0.8	1	0.6	0.6	0.6	0.8	0.8	1.2	***	D
LM14	0.6	0.8	0	0.6	0.6	0.8	0.6	0.8	***	F
LM15	0.6	1	0	0.8	0.6	0.8	0	0.8	**	D
LM16	0	1	0	1	0.6	1	0.8	1	**	F
LM17	0.8	1.2	0.6	0.8	0.7	0.7	0.6	0.8	***	D
LM18	0.7	1	0.6	0.8	0.6	0.6	0.8	0.8	***	F
LM19	0.6	0.9	0.6	0.6	0.8	0.8	0.7	0.8	***	F
LM20	0.7	1.1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM21	0.7	0.8	0.7	0.7	1	1	0.6	0.8	***	M
LM22	0.6	0.8	0.6	0.6	0.8	1	0.8	1	***	F
LM23	0.7	1	0.6	0.8	0.6	0.6	0	0.7	***	D
LM24	0.6	0.9	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM25	0.6	0.8	0.6	1	0.8	0.7	0	1	***	F
LM26	0.7	0.8	0.6	0.7	0.8	0.6	0.7	1	***	M
LM27	0.8	1.3	0.8	0.8	0.6	0.8	0.6	1	***	D
LM28	0.7	0.8	0.6	0.6	0.6	0.7	0.6	0.8	***	M
LM29	0	0.8	0	0.6	0.6	0.8	0	1	*	M
LM30	0.6	1	0.6	0.8	0.6	0.6	0.6	1	***	PF
LM31	0.6	0.8	0.6	0.7	0.6	0.6	0.6	0.8	***	M
LM32	0.6	1	0	0.6	0.6	0.6	0.6	0.8	***	F
LM33	0	1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM34	0.6	0.6	0.6	0.6	0.6	0.8	0.6	0.8	***	F
LM35	1	1	0	0.8	0.8	1.2	1	1.4	***	V
LM36	0.8	1	0.8	0.8	0	0.8	0.8	1	***	F
LM37	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM38	1.2	1	0.7	0.7	0.6	0	0	0.8	**	D
LM39	0.8	0.8	0.7	0.7	0.8	0.8	0.6	1	***	D
LM40	0.6	1	0	0.6	0.6	0.6	0.6	1	***	D
LM41	0.7	1.2	0.8	0.6	0.6	0	0.7	1	***	F
LM42	0	0.6	0.6	0.6	0.6	0.8	0	0.8	**	M
LM43	0.6	1.2	0.6	0.8	0.6	0.6	0	0.8	***	D
LM44	0.6	0.8	0.6	0.6	0.6	0.8	0.7	1	***	M
LM45	0.8	0.6	0.6	0.6	0.6	0.6	0.6	0.8	***	PF
LM46	0.7	1	0.6	0.7	0.6	0.8	0.6	1	***	F
LM47	0.6	1.2	0.6	0.8	0.6	0.8	0.6	1	***	M
LM48	0.6	1.2	0.6	0.6	0.8	0.8	0.8	1	***	D
LM49	0.6	1	0.6	0.6	0.7	0.8	0.6	0.8	***	M
LM50	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM51	1.2	1.3	0.8	0.8	0.6	0.6	0.6	0.8	***	M
LM52	0.6	1	0.6	0.6	0	0.6	0.6	0.7	***	F
LM53	0.6	0.9	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM54	0.9	1	0.6	0.6	0.6	0.8	0	0.8	***	M
LM55	0.6	0.8	0.7	0.6	0.6	0.6	0.6	0.8	***	M
LM56	0.6	0.8	0.6	0.6	0	0.6	0	0.8	**	D
LM57	1	1	0.7	0.6	0.6	0.8	0	1.1	***	D
LM58	0.6	0.8	0.6	0.7	0.7	0.6	0.6	0.8	***	M
LM59	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM60	0.6	1	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM61	0.9	1	0.9	0.6	0.6	0.6	0.6	0.9	***	PF
LM62	0.6	0.6	0.8	1	0.6	0.6	0.6	1.2	***	D
LM63	0.6	1	0.6	0.6	0	0	0	1	*	F
LM64	0.6	1	0.8	08	0.8	0	0	0.9	**	F
LM65	0.6	1	0.6	0	0.6	0.6	0	0.8	**	PF
LM66	0.6	0.8	0.6	0.6	0	0	0	0.8	*	F
LM67	0.6	0.6	0.6	0.6	0.6	0.7	0.6	0.9	***	F
LM68	0.6	0.6	0	0.6	0	0	0	0	ns	F
LM69	1	1.2	0.8	1	1	1.1	0.8	1.4	***	D
LM70	0.8	0.6	0.8	0.6	0.6	0.8	0.6	1	***	V
LM71	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM72	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM73	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM74	0.9	1.1	0.8	0.8	0.8	0.8	0.6	0.8	***	D

[a] Results indicate mean value of four determinations (carried out in duplicate and repeated twice). The values are expressed in cm. [b]P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001.

[c] M, Meat; D, Dairy; F, Fish; V, Vegetable; PF, Packaged food; CECT, Colección Española de Cultivos Tipo (http://www.cect.org/).

In general, the antibacterial effects of citrus EOs depend on the compounds and the species/isolate under study (Fisher *et al.*, 2008) and similar results, in terms of number of strains inhibited and inhibition areas, were previously registered for EOs extracted from Citrus in Sicily (south Italy) (Settanni *et al.*, 2012 and 2014). It is worth noting that in those previous studies, *L. monocytogenes* resulted the species most sensitive among the bacteria tested which included Gram-positive (*Staphylococcus aureus*), as well as Gram-negative (*Salmonella* spp. and *Enterobacter* spp.) strains.

MICs were calculated only for the most effective EOs (EO L2 and EO L8) against *L. monocytogenes* LM35 and LM69, which were registered as the most sensitive strains. Both strains were equally inhibited and the values registered were 0.625 µL/ml for EO L2 and 1.25 µL/ml for EO L8. The two strains LM35 and LM69 were chosen to be better characterized and then used to register their behavior in edible film formulations. In our opinion, the best strategy to evaluate the efficacy of the incorporation of a given EO in films should be based on the use of the most sensitive strains. In fact, the in situ activity can be strongly reduced by the interaction of the EOs with the films and the inhibitory effect on the test strains masked.

2.2.2. Characterization of EOs by GC/MS

Analysis of volatile compounds was carried out after extraction of EOs. Based on the antilisterial activity, EO L2 and EO L8, as most effective, and EO O3 and EO M7, as less effective oils, were chemically analyzed by GC/MS.

The identified volatile compounds and their relative amounts are given in Table 2.3. A total of 36 compounds were characterized among the four EOs. The phytochemical groups included monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons.

Table 2.3. Chemical composition[a] of citrus EOs.

Compound	**RT**	**EO L2**		**EO O3**		**EO M7**		**EO L8**		**Statistical significance[b]**
Monoterpene hydrocarbons		88.35		98.07		97.81		90.93		
α-Thujene	9.801	0.215	B	n.d.	A	n.d.	A	0.305	C	***
α-Pinene	10.129	1.290	B	0.340	A	0.410	A	1.325	B	***
Sabinene	11.900	1.105	B	0.210	A	0.220	A	1.135	B	***
β-Pinene	12.155	9.890	C	0.025	A	0.025	A	9.125	B	***
β-Myrcene	12.666	1.105	A	1.695	C	1.890	D	1.425	B	***
α-Phellandrene	13.467	0.185	C	0.105	B	0.055	A	0.065	A	***
3-Carene	13.560	n.d.	ns	0.090	ns	0.040	ns	n.d.	ns	ns
α-Terpinene	13.944	n.d.	ns	0.040	ns	0.040	ns	0.340	ns	ns
p-Cymene	14.275	11.515	C	n.d.	A	n.d.	A	0.440	B	***
D-Limonene	14.854	62.780	A	95.445	C	94.910	C	64.505	B	***
ϒ-Terpinene	16.080	0.025	A	0.075	A	0.180	B	9.525	C	***
(+)-2-Caren	17.315	n.d.	A	0.045	B	0.035	B	0.510	C	***
cis-2,6-Dimethyl-2,6-octadiene	29.716	0.240	B	n.d.	A	n.d.	A	2.225	C	***
Oxygenated monoterpenes		10.770		1.930		2.175		8.275		
1-Octanol	16.736	0.065	C	n.d.	A	n.d.	A	0.050	B	***
Linalol	18.024	0.425	A	1.005	B	1.555	C	0.410	A	***
Nonanal	18.252	0.190	B	0.040	A	0.020	A	0.135	B	**
Fenchol	18.972	0.030	B	n.d.	A	n.d.	A	0.015	B	**
Limonene epoxide	19.608	0.815	B	n.d.	A	n.d.	A	n.d.	A	***
Limonene oxide, trans	19.820	1.000	ns	n.d.	ns	n.d.	ns	n.d.	ns	ns
β-Terpinol	20.507	n.d.	A	0.035	C	0.020	B	n.d.	A	**
Citronellal	20.556	0.065	B	n.d.	A	n.d.	A	0.095	C	***
4-Terpineol	21.971	0.630	B	0.235	A	0.225	A	1.010	C	***
α-Terpineol	22.705	1.445	D	0.415	B	0.265	A	1.100	C	***
Decanal	23.157	0.085	A	0.200	A.C	0.090	A	0.040	A,B	*
trans-Carveol	23.801	0.180	B	n.d.	A	n.d.	A	n.d.	A	***
cis-Geraniol	24.087	0.175	B	n.d.	A	n.d.	A	1.245	C	***
α-Citronellol	24.200	0.070	A	n.d.	A	n.d.	A	0.325	B	**
β-Citral	24.704	1.550	C	n.d.	A	n.d.	A	1.355	B	***
(-)-Carvone	24.947	0.165	B	n.d.	A	n.d.	A	n.d.	A	***
cis-p-Mentha-2,8-dien-1-ol	26.058	0.220	B	n.d.	A	n.d.	A	1.790	C	***
α-Citral	26.090	1.980	B	n.d.	A	n.d.	A	n.d.	A	***
Geranyl acetate	30.116	0.980	C	n.d.	A	n.d.	A	0.325	B	***
Neryl acetate	30.979	0.700	C	n.d.	A	n.d.	A	0.380	B	***
Sesquiterpene hydrocarbons		0.880		n.d.		0.020		0.800		
α-Bergamotene	33.375	0.315	C	n.d.	A	n.d.	A	0.275	B	***
β-Bisabolene	36.474	0.480	C	n.d.	A	n.d.	A	0.385	B	***
Caryophyllene oxide	39.463	0.085	C	n.d.	A	0.020	B	0.140	D	***

[a] Data are means of two replicates expressed as percent area.[b] P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001. Abbreviations: RT, retention time on SLB-5MS column; ns, not significant; n.d., not detectable.

Monoterpene hydrocarbons were quantitatively relevant, ranging from 88.35% (EO L2) to 98.07% (EO O3). Limonene accounted for the major proportion by quantity in all samples. The oxygenated monoterpenes of lemon EOs were four/five folds those of EO O3 and EO M7, indicating a direct role in the mechanisms of inhibition. Sesquiterpene hydrocarbons were detected in minimal percentages in lemon EOs, only traces were found in EO M7 while they were absent in EO O3.

Monoterpene hydrocarbons such as a-Thujene, p-Cymene and cis-2,6-Dimethyl-2,6-octadiene were found only in lemon EOs. Among the oxygenated monoterpenes, 1-Octanol, Fenchol, Citronellal, cis-Geraniol, a-Citronellol, b-Citral, cis-p-Mentha-2,8-dien-1-ol, Geranyl acetate and Neryl acetate were identified only in EO L2 and EO L8. On the contrary, b-Terpinol was only found in EO O3 and EO M7. Almost all compounds showed statistical differences in quantitative terms among EOs. The higher presence of oxygenated monoterpenes in volatile composition profile of EO L2 and EO L8 could explain the greater inhibitory activity than the EO O3 and EO M7.

2.2.3. Viability assay

Dead and viable cells were detected and counted using epifluorescence microscopy. Some pictures of live and dead cell exposed to the EOs are reported in Figure 2.2. Plate counts of the untreated samples showed an increase of 10^3 CFU/ml for both strains within the six hours of treatment. Divergent results were obtained comparing the counts assessed by epifluorescence microscopy and plate counts. Based on epifluorescence microscopy, viable cells amounted to 10^{3-4} CFU/ml for LM35 and

10^{4-5} CFU/ml for LM69, while dead cells reached up to 3 and 4 log CFU/ml in case of LM35 and LM69, respectively. These results are in contrast with those of direct plate counts, where no cultivable cells were detected after 1 h (or 2 h in case of LM35 added with EO L8) of incubation. This could be explained by an active but non-culturable (ABNC) state of cells stressed by EOs (Boulos *et al.*, 1999). This was confirmed by Nexmann *et al.* (1997) who registered significantly fewer viable *L. monocytogenes* cells counted by culture-based techniques compared to the active bacteria detected using fluorescent direct counts. Similar results were achieved with lactic acid bacteria (Moreno *et al.*, 2006) using fluorescent flow cytometric measurements (Boulos *et al.*, 1999).

According to Joux *et al.* (2000), bacterial cells cannot be necessarily considered active if they show intact membranes, but it would seem to be more accurate to assume that membrane–compromised cells are dead (Berney *et al.*, 2006). The EOs antimicrobial activity is due to their hydrophobic nature affecting the lipid bilayer of microbial cells, as confirmed by the evidences of this assays, since the kit used enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, differentiating between active and dead cells (Sachidanandha *et al.*, 2005).

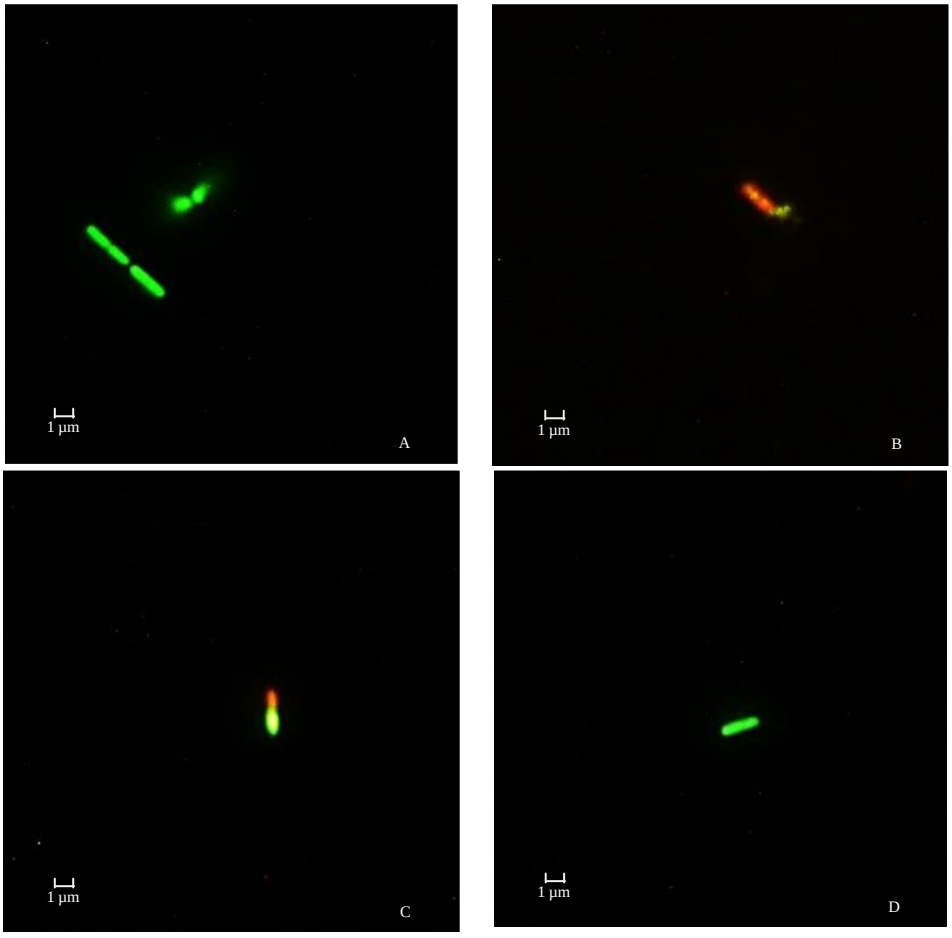


Figure 2.2. Live (green) and dead (red) cells observed by fluorescence microscopy. A, Control, live cells; B, EO , dying cell; C, EO, dead and live cells; D, live cell.

2.2.4. Antilisterial effect of edible EOs-based films and film microstructure

Antilisterial performances of CH- and MC-based edible films determined on TSA, alone and in combination with EO L2 and EO L8, are shown in Figure 2.4. The overall effect of CH- and MC-based films, in terms of trend, was similar for both strains tested. The addiction of the EOs into the films enhanced their bactericidal activity. The highest antimicrobial effect was obtained for CH films at 8°C (Fig. 2.4 E and G). When sample EO L2 was added to the films, a reduction in the range of 2–3 Log CFU/cm^2 was obtained as compared to control plates (Fig. 2.4 A and E).

This oil sample determined the lowest *Listeria* counts in both film matrices (CH or MC). In general, the EO L2-based films showed the best inhibition activity compared with the CH or MC control films, and also, compared to EO L8-based films.

After a storage period of 24 h at 37°C and 7 days at 8°C, pure MC films showed no significant effect on the growth of both strains. MC films incorporating EO L2 promoted a slight reduction in Listeria counts at 37°C after 8 h of incubation (<1–2 log CFU/cm^2) (Fig. 2.4 B). A stronger antilisterial effect was evidenced for the CH-based films, alone and in combination with EOs. Specifically, CH-films were more effective in reducing the microbial growth at 8°C rather than 37°C. In fact, CH-films added with EOs led to a reduction up to 3 and 6 log CFU/cm^2, in the case of LM35 and LM69, respectively, when incubated at 8°C for 7 days (Fig. 2.4 E and G). The highest significant antibacterial effect evidenced in case of the incubation at 8°C may be related to the influence of the temperature in promoting the permeability of cell membranes and, thus, dissolving more easily EOs in the lipid bilayer when low temperatures occur (Sanchez-Gonzalez *et al.*, 2011). Fig. 2.2 shows the SEM microstructures of the cross-sections of CH and MC films. Pure MC and CH films (Fig. 2.4 A and D) exhibited a homogeneous and continued microstructure in line to that observed in previous studies (Vargas *et al.*, 2011). The addition of the lemon EOs to the film matrix promoted discontinuities (Fig. 2.4 B, C, E and F), in agreement with the results reported by Perdones *et al.* (2012) in CH-based films containing essential oil. The presence of EO droplets is more noticeable in CH-based films (Fig. 2.4 B and C), and especially in films containing EO L2 (droplets size 1–8 mm).

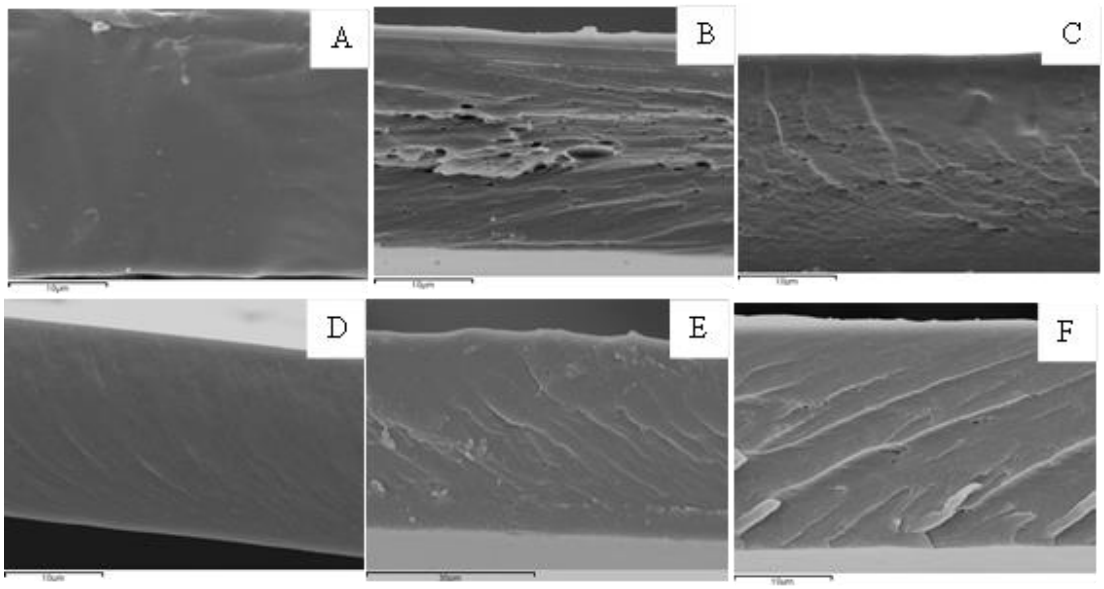


Figure 2.3. SEM microstructure of cross sections of chitosan and methylcellulose films with essential oils. Magnification is x3500. A, chitosan films; B, chitosan film with EO L2; C, chitosan film with EO L8; D, methylcellulose film; E, methylcellulose film with EO L2; F, methylcellulose film with EO L8.

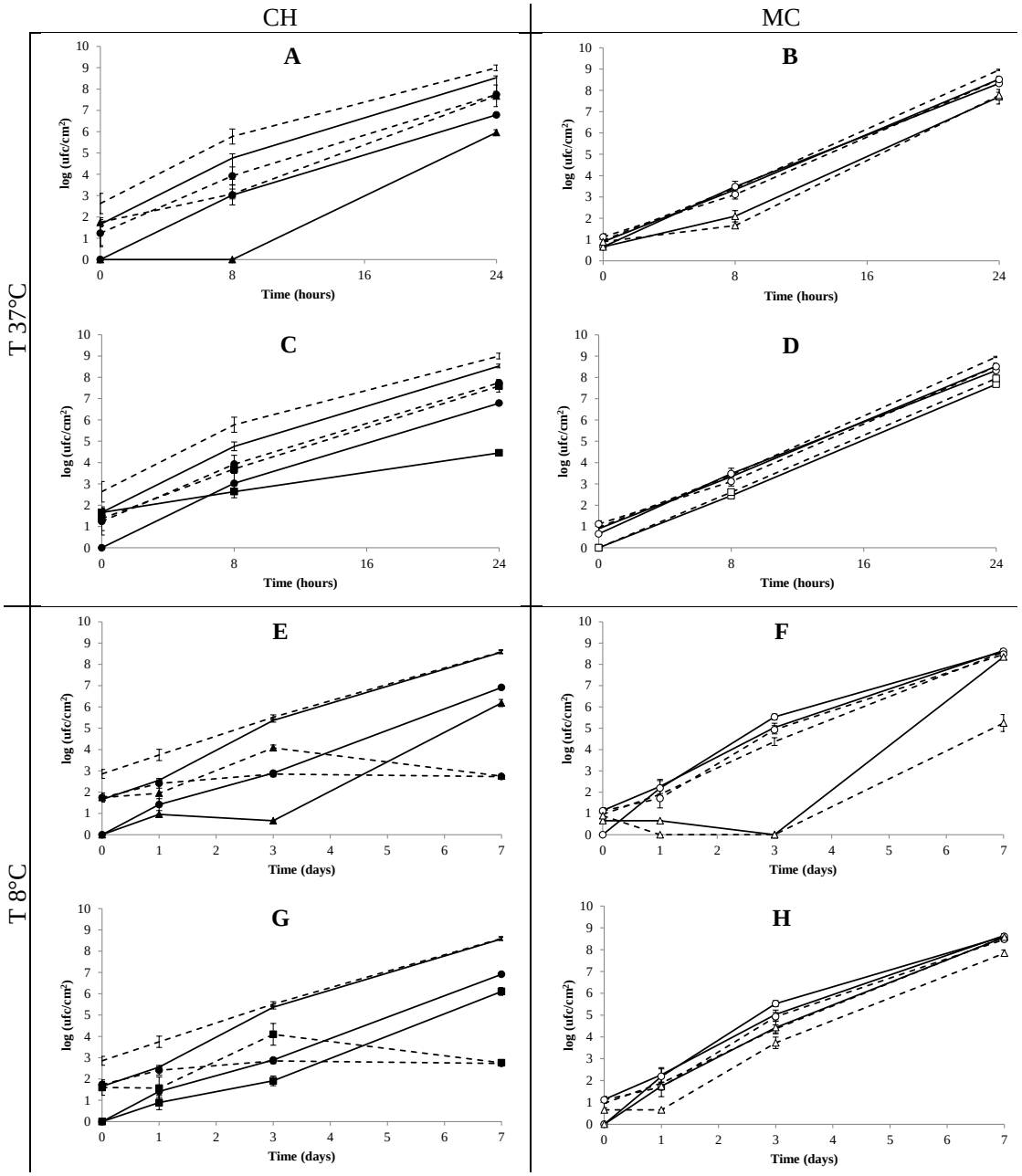
The observations pointed to a better incorporation of the EOs in CH matrix, where a higher amount of oil droplets was distinguished. Furthermore, the higher inhibition activity recorded for EO L2 included into CH matrix can be due not only to the better incorporation, but also to the subsequent release of the active compounds.

A good incorporation of EO into the films slows down the diffusion rate of the antimicrobial compounds, keeping high concentrations of EOs for extended period of time and reducing the levels of microorganisms on the surface.

The two strains LM35 and LM69 chosen to evaluate the efficacy of the inclusion of EOs in films had different food origin, specifically vegetable and dairy products, respectively. Thus, this study demonstrated the potential application of the EOs to inhibit *L. monocytogenes* from different sources. Although the resistant strains will

not be inhibited by this strategy, a strong reduction of this pathogen can be obtained in terms of sensitive strains.

Figure 2.4. Effect of incorporation of EOs in chitosan and methylcellulose films on the growth of *L. monocytogenes* at 37°C for 24 h (A, B, C and D) and 8°C for 7 d (E, F, G and H).



Symbols:—, strain LM35; – –, strain LM69; black marks indicate chitosan films; empty marks indicate methylcellulose films; unmarked lines indicate control strains; ●,○, indicate control films; ▲, Δ, indicate films with EO L2; ■, □, indicate films with EO L8. A and E, chitosan films with EO L2; B and F, methylcellulose films with EO L2; C and G, chitosan films with EO L8; D and H, methylcellulose films with EO L8.

2.3. Conclusions

Citrus EOs showed bioactive properties against *L. monocytogenes*. The efficacy of the inclusion of EOs in films was tested against the most sensitive strains, in order to better evaluate their suitability. A masking effect of the film matrices on the inhibitory properties of the active substances cannot be excluded and could be relevant determining negative results in presence of low sensitive strains. The antibacterial effect of the EOs showing the highest inhibitory power was maintained when they were incorporated into biodegradable films based on chitosan or methylcellulose.

Chitosan films containing EO L2 were the most effective in reducing *L. monocytogenes* counts. Chitosan edible films enriched with lemon oils represent an alternative tool to control surface contaminations of *L. monocytogenes*, especially in refrigerated conditions. The reduction in EO concentration needed for film applications, as compared to direct contact treatments, can reduce the possible sensory impact on food. Works are being prepared to refine the technology for the production of EO-based films, to evaluate the suitability of the films tested in this study on food matrices, as well as the impact of the EO released on the sensory quality. Hence, the foreseeable potential practical application of this study is to reduce the presence of *L. monocytogenes* in foods, but also to valorise citrus fruit peel that basically constitutes awaste of the fruit juice industry in Sicily.

Chapter 3

Chemical composition and antimicrobial activity of essential oil extracted from the peel of a Sicilian mandarin cv. Tardivo di Ciaculli

3.2. Materials and Methods

3.2.1. EO extraction and characterization

The EO analyzed in this study was obtained from peels of Tardivo di Ciaculli mandarin (Figure 3.1). Peels were immediately subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Comandè, Palermo, Italy) collecting the oil in hexane. EO was dried over anhydrous sodium sulphate and stored at 4°C in air-tight sealed glass vials covered with aluminum foil prior to be used in chemical and microbiological analysis.

Figure 3.1. Peeling, hydro-distillation and gas chromatography/mass spectrometry characterization of Tardivo di Ciaculli mandarin EO.

EOs chemical characterization was carried out by gas chromatography/mass spectrometry (GC/MS) (EI) using a a GCMS-QP2010 (Shimadzu, Milan, Italy). For analysis of the EOs samples, 1.0 ml of solution (1:10 v/v, essential oil/hexane) was placed in injection port. GC and MS scan conditions are previously reported in detail

(Settanni *et al.*, 2014). Data were handled through the use of GCMS-Solution software and the peak identification was carried out with NIST21,107,147 Library according to a similarity larger than 90% and other published mass spectra. GC/MS analysis was carried out in duplicate. A commercial mandarin EO was included in the characterization for comparison.

3.2.2. Bacterial strains

Twenty different foodborne pathogen strains were tested for their sensitivity to EO. All the strains were provided by the Department of Sciences for Health Promotion and Mother-Child Care "G. D'Alessandro" – University of Palermo (Palermo, Italy) and belonged to *Listeria monocytogenes* (5 strains), *Staphylococcus aureus* (5 strains), *Salmonella enterica* (5 strains) and *Enterobacter* spp. (5 strains). Bacterial strains were sub-cultured in Brain Heart Infusion (BHI) agar (Oxoid, Milan, Italy) and incubated overnight at 37°C.

3.2.3. Antibacterial activity

A modified paper disc diffusion method (Militello *et al.*, 2011) was applied to test the antibacterial activity of the mandarin EOs. It is described in deteails in Chapter 1. Each test was performed in duplicate and the experiments were repeated twice in two consecutive days.

3.3. Results and discussion

The GC-MS analysis identified compunds belonging to monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons.

The analysis showed only quantitative differences between the commercial and Tardivo di Ciaculli mandarin EOs (Table 3.1).

Table 3.1. Percentage contribution of the main chemical classes characterizing mandarin EO extracted from peels.

	Commercial EO	**Tardivo di Ciaculli EO**
Monoterpene hydrocarbons	95.37	94.52
Oxygenated monoterpenes	1.39	3.66
Sesquiterpene hydrocarbons	0.26	0.15
Others	0.55	1.67

Among the monoterpene hydrocarbons, representing about the 95% of the EOs, the main compounds were D-limonene, γ-terpinene, terpinolene, β-pinene and β-mircene. D-limonene was the compound at highest concentration in both the EOs analized (Figure 3.2 A).

α-terpineol, 4-terpineol, thymol, geranial and linalool were the main compounds among the oxygenated monoterpenes that showed a variable concentration between 1 and 4% (Figure 3.2 B). These compunds have been already indicated as responsible of antimicrobial activity, even if at different extent (Settanni *et al.*, 2012 and 2014).

Regarding the bacterial inhibition, both EOs showed higher activity against Gram-positive, such as *Listeria monocytogenes* and *Staphylococcus aureus*, than Gram-negative, as *Salmonella enterica* and Enterobacter spp (Table 3.2).

Table 3.2. Antimicrobial activity of commercial and Tardivo di Ciaculli EOs.

	Strain	Commercial EO	Tardivo di Ciaculli EO
Gram +	*Listeria monocytogenes* 133	±	-
	Listeria monocytogenes 140	-	±
	Listeria monocytogenes 180	-	++
	Listeria monocytogenes 182	-	+++
	Listeria monocytogenes 14 BO	-	±
	Staphylococcus aureus C38/249,1-MSSA	-	+
	Staphylococcus aureus 340-MRSA	-	+
	Staphylococcus aureus 68 MRSA	-	±
	Staphylococcus aureus 156 MRSA	-	+++
	Staphylococcus aureus 637 MRSA	-	+
Gram -	*Salmonella enterica* 50398	-	-
	Salmonella enterica 50374	-	-
	Salmonella enterica 50399	+	-
	Salmonella enterica 50339	+	+
	Salmonella enterica 50272	++	±
	Enterobacter hormaechei 2	±	-
	Enterobacter hormaechei 11	±	±
	Enterobacter hormaechei 19	±	+
	Enterobacter cloacae 24	-	+
	Enterobacter cloacae 13A	-	+

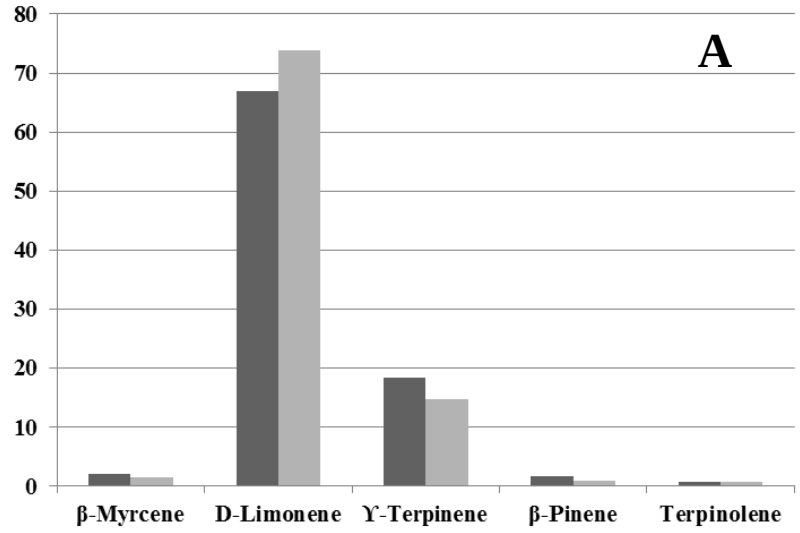


Figure 3.2. Quantitative differences of monoterpene hydrocarbons (A) and oxygenated monoterpenes (B) between commercial (dark grey) and Tardivo di Ciaculli EOs (light grey) (expressed as percentage of area resulting from GC-MS analysis).

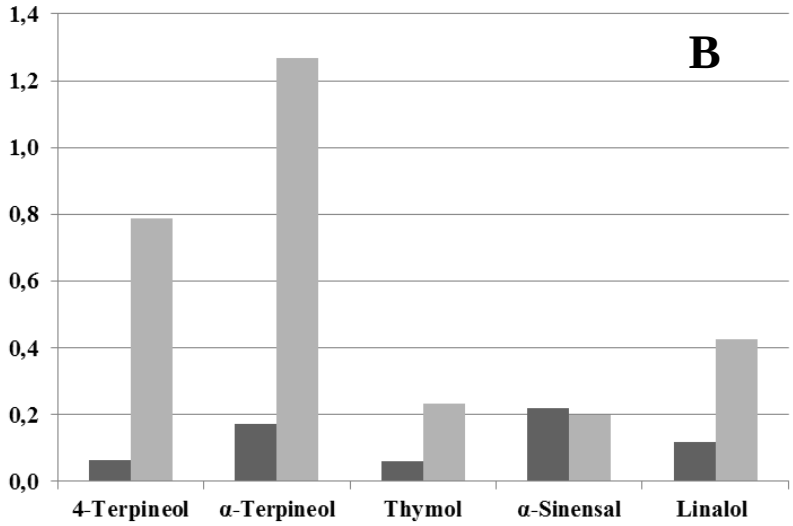


Figure 3.2. Continue.

The higher amount of oxygenated monoterpenes in Tardivo di Ciaculli EOs could explain the higher antibacterial activity compared to the commercial one.

The Tardivo di Ciaculli EO could represent a possible option for food preservation purposes.

Chapter 4

Quantitative detection of viable foodborne E. coli O157:H7 combining propidium monoazide and real-time PCR after inactivation by Citrus essential oils

4.2. Materials and Methods

4.2.1. Bacterial strains, culture conditions and DNA isolation

The *E. coli* O157:H7 CECT 5947 (non-toxigenic) supplied by the Spanish Type Culture Collection (CECT, http://www.cect.org/) was used in this study. This strain is recommended for food control since gene *stx2* (virulence factor) has been replaced with gene *cat*. *E. coli* was routinely grown on Tryptic Soy Broth (TSB) at 37°C for 18h, and enumerated by plate count on Trypticasein Soy Agar (TSA), at the same incubation conditions. Inocula for antibacterial tests were prepared by transferring 100 µl of the overnight culture to 10 ml of TSB and incubated at 37°C for 2 h (ca. 10^8 CFU/ml). Thereafter cultures were serially diluted in Phosphate Buffered Saline (PBS) to obtain a final desired cell density. DNA was purified using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany) according to the manufacturer's instructions.

4.2.2. Essential oils

Two EOs were extracted by hydrodistillation from the peels of two Sicilian lemon cultivars: EOL2 derived from cv. Femminello Santa Teresa while EOL8 derived from cv. Limone KR Siracusano. They were previously chemically characterized by GC/MS and tested for their antimicrobial activity (Settanni *et al.*, 2014; Randazzo *et al.*, 2016).

4.2.3. MIC determination and kinetics of inactivation

The antibacterial activity of the EOs against *E. coli* O157:H7 CECT 5947 strain was firstly confirmed by the paper disc diffusion method applied by Militello et al. (2011). To determine the MIC, serial dilutions of each EO (dilution factor = 2) were obtained with dimethyl sulfoxide (DMSO, SigmaeAldrich, Milan, Italy). 10^5 CFU/ml of *E. coli* broth cultures were added with EOs serial dilutions. The evaluation of inhibition was perfomed by visual inspection after 24 h and confirmed after 48 h. To evaluate the kinetics of inactivation, *E. coli* cultures of ca. 10^8 CFU/ml were prepared by diluting a 2 h culture on TSB. EOs were then added to culture broths at a concentration of 5 µl\ml, the same as resulting from MIC determination. They were further incubated at 37°C in a shaker and samples were taken at 0, 30 min, 1 h, 2 h and 4 h. Samples were spread on TSA plates for plate counts and the optical density was as well measured. All experiments were independently repeated three times.

4.2.4. PMA cross-linking

Detection of live cells was carried out using a PMA treatment as described by Elizaquivel *et al.* (2012). Briefly, 20mM PMA stock solution were added to 500 µl of either viable or EO-treated cells at a final concentration of 100 µM that had proved non-toxic for live cells (Elizaquivel *et al.*, 2012). Each sample was treated in triplicate to ensure reproducibility of results. After the addition of PMA, samples were incubated for 5 min in the dark, at room temperature, with occasional mixing to allow reagent penetration. Thereafter, samples were exposed to light for 15 min using a photo-activation system (Led-Active Blue, Geniul, Barcelona, Spain). After

photoinduced cross-linking, cells were centrifuged at 7000 rpm for 5 min and supernatant was removed. The resulting pellets were used for DNA isolation.

4.2.5. Real-time PCR quantification

Primer sequences were uidAR383-ACC AGA CGT TGC CCA CAT AAT T and uidAF241-CAG TCT GGA TCG CGA AAA CTG and the probe uidAP266-NED-ATT GAG CAG CGT TGG-NFQ. PCR reactions were performed in a final volume of 20 µl, containing 10 µl of 2× Brilliant® II QPCR Master Mix with high ROX (Stratagene, Madrid, Spain) and 5 µl of template DNA. Concentrations of primers and probe were 250 nM of each primer and 25 nM of the uidA probe. All amplifications were performed on the LightCycler 480 System (Roche Diagnostics, Mannheim, Germany). The standard protocol included one cycle at 95°C for 15min, followed by 40 cycles at 95°C for 15 s, and 63°C for 1 min. Fluorescence was measured at the end of each extension step. Reactions were done in duplicate. In all cases a negative control of amplification was included using 5 µl of water instead of DNA template.

Standard curve was generated using 10-fold serial dilutions of DNA extracted from *E. coli* O157:H7 CECT 5947 covering the range from 10^0 to 10^4 cfu/ml per reaction, calculated on the basis of the genome size of this pathogen (Hayashi *et al.*, 2001). The crossing point (Cp) values obtained from the assay of each dilution were used to plot a standard curve by assigning the corresponding concentration values by using Roche LightCycler® 480 SW 1.5 software.

4.3. Results

4.3.1. MIC determination and kinetics of inactivation

EO L2 and EO L8 inhibited *E.coli* O157:H7 CECT 5947 by the paper disc diffusion since halos of inhibition were detected. The MIC resulted of 5 µl\ml for both EOs. The kinetics of inactivation of EOs tested for killing *E. coli* O157:H7 as determined by plate count is shown in Figure 4.1. For both EOs, a decrease in bacterial counts was observed after 30 min and still up to 4 h. After 6 h of incubation, the remaining population grew raising the bacterial counts. The highest reductions registered were for EO L8 reaching 7 log of reduction after 4 h of incubation.

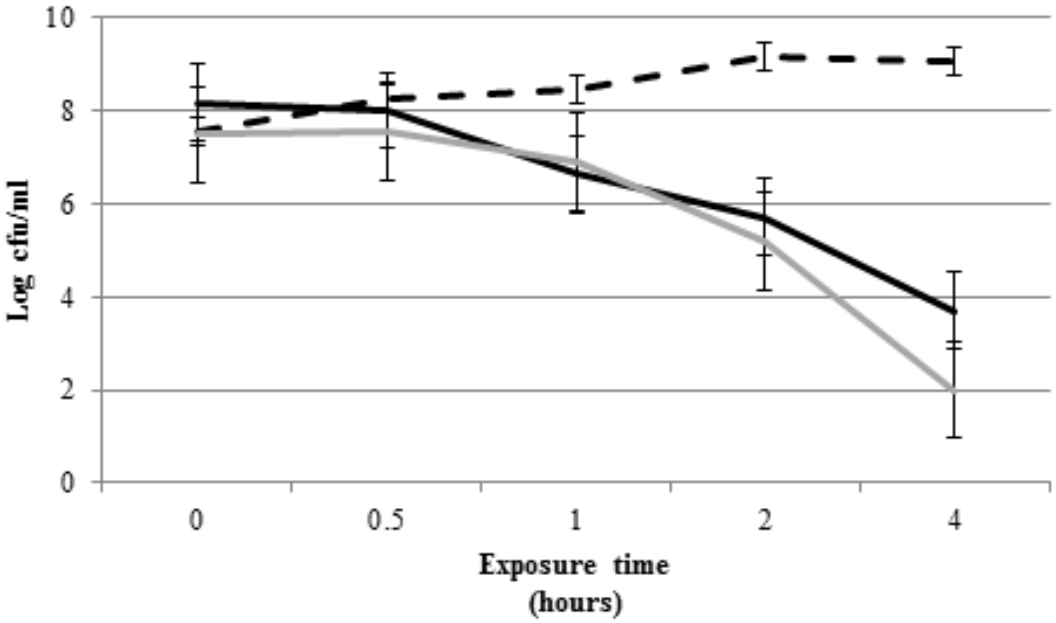


Figure 4.1. Kinetics of inactivation of EO L2 (black) and EO L8 (grey) against *E. coli*. Untreated sample represent the control (dotted black line).

4.3.2. PMA cross-linking and real-time PCR quantification

In order to test the ability of the PMA-qPCR procedure to monitor *E. coli* O157:H7 inactivation by EOs, cultures of 10^9 CFU/ml were treated 4 h with 10µL/ml of each

of the two EOs. Following, they were quantified by qPCR with and without previous PMA treatment by using the standard curve obtained from purified *E. coli* O157:H7 DNA (y=−3.545×+26.26; efficiency=1.914). Quantification derived from qPCR determination of PMA-treated and non-treated samples showed that population decreased 2 log orders (Table 4.1), while comparing live and EOs+PMA treated cells 4 log of reduction were reported. These last results showed 1 log discrepancy if compared with those achieved by plate counts.

Table 4.1. Comparison between qPCR and direct counts obtained from *E. coli* O157:H7 treated with EOs.

Sample	qPCR quantification		Plate counts (Log CFU/ml)
	Cp value	Log CFU/ml	
E.coli live	18.83±0.62	9.15	9.13
E.coli live + PMA	18.61±0.03	9.19	-
E.coli EO2 treat	24.92±0.24	7.41	4.40
E.coli EO2 + PMA treats	32.19±0.33	5.37	-
E.coli EO8 treat	25.88±0.01	7.14	4.33
E.coli EO8 + PMA treats	33.36±0.59	5.05	-

4.4. Discussion

Enterohemorrhagic *E. coli* (EHEC) particularly O157:H7 is one of the most investigated foodborne pathogens due to the severity of the disease. Outbreaks can infect thousands of people causing bloody diarrhea and hemolytic uremic syndrome (HUS) that can result in severe illness or even death (Chattaway *et al.*, 2011). In verified outbreaks, where the causative agent was known, pathogenic *E. coli* accounted for 1.92% of the outbreaks (EFSA, 2015). It is commonly found in the intestines of warm-blooded animals and its presence in food is considered as a

possible faecal contamination and\or as an inadequate hygiene practices (Ragaert *et al.*, 2011), involving meat, fish, vegetable and fruit preparations.

In this work, two lemon EOs (EO L2 and EO L8) were effective in inhibiting *E. coli* O157:H7, showing MIC of 0.5%. Further on, we assessed this inhibition activity by using a novel quantitative method based on qPCR coupled with a PMA treatment. This procedure was able to discriminate between live and dead cells resulting after EOs treatments. PMA treated live cells showed amplification levels similar to those obtained from non-treated cells (9.15 and 9.19 Log CFU/ml, respectively) demonstrating that PMA treatment did not affect live cells nor the efficiency of DNA amplification in the reaction. Moreover, these results are in line with those detected by plate count (9.13 Log CFU/ml).

Different quantification values were detected for live and EOs-exposed cells without PMA pre-treatment (9.15, 7.41 and 7.14 Log CFU/ml *E. coli* live, *E. coli* EO2 treated and *E. coli* EO8 treated, respectively). This indicates the loss of DNA from dead cells during the extraction procedure.

Comparing the quantification results of PMA-treated EOs exposed cells (5.37 and 5.05 log CFU/ml corresponding to *E. coli* EO2+PMA and *E. coli* EO8+PMA, respectively) with those of untreated EOs exposed cells (7.41 and 7.14 log CFU/ml *E. coli* EO2 and *E. coli* EO8, respectively), differences of about 2 log CFU/ml were reported. Previous studies suggested that treatment with EOs might stress bacterial cell in a sub-lethal manner, leading to a non-cultivable state that may be reversible (Randazzo *et al.*, 2016; Blatchley *et al.*, 2007). The cell membranes of these organisms may still be impermeable to penetration by PMA and thus not allow

inactivation of their genomic DNA. All above considerations could explain the discrepancies between *E. coli* qPCR quantifications with and without PMA pre-treatment.

When samples were tested by PMA-qPCR, quantification values (5.37 and 5.05 log CFU/ml for *E. coli* EO2+PMA treatment and *E. coli* EO8+PMA treatment) were slightly higher than the expected (4.40 and 4.33 Log CFU/ml for *E. coli* EO2 treatment and *E. coli* EO8 treatment). These quantification discrepancies suggest that DNA from dead cells was not completely removed by PMA treatment. This was also observed by Elizaquível *et al.* (2012) and Varma *et al.* (2009) when high levels of biomass were used. Thus, high cell concentrations interfere with the ability of the PMA-qPCR method to detect live cells specifically. In fact, the effectiveness of PMA activity may be saturated by increasing cell numbers, at least under the treatment conditions employed in this study.

However, it is noteworthy to comment that such high bacterial pathogen concentrations are not usually found in naturally contaminated food products.

In addition, all these results achieved by PMA-qPCR confirm that the damage to *E. coli* cells due to EOs exposure occurs at a cell membrane level, since PMA is capable of penetrating only the compromised cell membranes of EOs treated cells.

Moreover, PMA pre-treatment of DNA extracts is effective in substantially reducing qPCR detectable target sequences from membrane-compromised cells of *E. coli*. Therefore, the PMA-qPCR procedure has the potential to specifically detect the presence of live cells among those exposed to EOs.

Chapter 5

Chemical compositions and antifungal activity of essential oils extracted from Citrus fruits cultivated in Sicily

5.2. Material and Methods

5.2.1. Fruit collection, EOs extraction and characterization

The EOs analyzed in this study were obtained from the peels of eight different citrus fruits cultivated in Sicily (Table 5.1). Fruits were collected on March 2014 from the "Parco d'Orleans" orchard of the Department of Agricultural and Forestry Science (Palermo, Italy) and from the "Azienda Sperimentale Palazzelli" C.R.A.-A.C.M. (Acireale, Italy). Citrus fruit peels were immediately subjected to hydrodistillation using a Clevenger-type apparatus. The EOs showing the highest (EO L1 and EO L2) and lowest (EO AM and EO MA) antifungal activities were analysed by gas chromatography/mass spectrometry (GC/MS), identifying the peaks according to NIST21,107,147 Library with a similarity of 90%, at least.

Table 5.1. Sicilian EOs used in antifungal screening.

EO	Species	Variety	Sperimental Orchard
MC	Mandarin (*Citrus reticulata* Blanco)	Mandarino Tardivo di Ciaculli	Campo dei Tigli (Palermo)
L1	Lemon (*Citrus limon* L. Burm.)	Femminello Santa Teresa	Campo dei Tigli (Palermo)
AM	Sweet Orange (*Citrus sinensis* L. Osbeck)	Moro Nucellare	Campo Palazzelli (Acireale)
AL	Sweet Orange (*Citrus sinensis* L. Osbeck)	Lane Late	Campo Palazzelli (Acireale)
AT	Sweet Orange (*Citrus sinensis* L. Osbeck)	Tarocco Tardivo	Campo Palazzelli (Acireale)
AS	Sweet Orange (*Citrus sinensis* L. Osbeck)	Sanguinello Nucellare	Campo Palazzelli (Acireale)
MA	Hybrid of Horoval clementine x Tarocco orange	Alkantara mandarin ®	Campo Palazzelli (Acireale)
L2	Lemon (*Citrus limon* L. Burm.)	Limone KR (Siracusano)	Campo Palazzelli (Acireale)

5.2.2. Antifungal activity

Forty-seven foodborne filamentous fungal strains of different species and origin (Table 5.2) were considered for preliminary antifungal assays. All strains, belonging

to the Dipartimento Scienze Agrarie e Forestali (Università di Palermo, Italy), were previously isolated from food matrices, mainly from honey (Sinacori *et al.*, 2014) and strawberries (La Scalia *et al.*, 2015). All the strains were sub-cultured onto Malt Extract Agar (MEA, Sigma-Aldrich) at 25°C for 5 or more days depending on strains' characteristics.

Antifungal activity was assayed by agar diffusion test (Lang *et al.*, 2012). Briefly, MEA plates were inoculated with approximately 10^4 spores/ml and a well for each plate was punched into the center of the agar layer. 20µl of EO was directly applied to the well and plates were left to incubate at 25°C. Halos, indicating fungal inhibition, were recorded dairy for up to 10 days, depending on the strain.

5.3. Results and Discussion

Based on the well diffusion assay, EO L1 and EO L2 showed a wide spectrum of activity, inhibiting 39 and 41 isolates, respectively, with halos of at least 10 mm of diameter. The EO AM and EO MA showed the lowest antifungal activity among the EOs tested in this study: their inhibition was <10 mm against 46 and 47 strains, respectively (Table 5.2).

Regarding the GC-MS analysis, a total of 36 compounds were identified and belong to three phytochemical groups: monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes hydrocarbons (Figure 5.1).

Table 5.2. Antifungal activity of Sicilian EOs assessed by well diffusion assay (inhibition halos in cm).

Fungal specie	**Strain**	**OE MC**	**OE L1**	**OE AM**	**OE AL**	**OE AT**	**OE AS**	**OE MA**	**OE L2**
Alternaria alternata	F 2.31	0.8	1	0.5	0.5	-	0.5	-	1
Alternaria tenuissima	F 1.8	0.5	0.8	-	-	-	0.5	-	1
Aspergillus niger	ML 111 B	1	3.5	-	1.2	0.8	1.2	-	4
Aspergillus niger	ML 113	0.8	2.5	-	1	0.5	0.8	-	1.8
Aspergillus niger	ML 168 A	-	1.4	-	-	-	0.5	-	0.8
Aspergillus niger	ML 168	-	1.5	-	0.5	-	0.5	-	1.4
Aspergillus niger	ML 168 B	0.8	2.5	-	1.4	0.5	1.5	-	2
Aspergillus niger	CC22A1	0.5	3	0.5	1	-	0.8	-	3
Aspergillus proliferans	ML 280	1	3	1	1.2	-	0.5	0.5	2.5
Aspergillus spelunceus	ML 442	0.5	1	-	0.5	0.5	0.5	-	1.6
Botritis cinerea	SANDRA01	1	2.5	0.5	1	1	1	-	2.5
Botryotinia fuckeliana	F 1.10	0.5	1.5	-	0.5	1	1	0.5	1.2
Chaetomium globosum	ML 176	0.5	0.8	0.5	0.5	0.5	-	-	1.4
Cladosporium cladosporioides	F 2.11	1.5	2	0.5	0.5	0.5	0.5	-	1.6
Daldinia concentrica	ML 286	-	2.5	0.5	-	-	-	-	
Daldinia concentrica	ML 287	-	1.5	-	1.2	-	0.5	-	1.8
Emericella discophora	ML 297	-	0.5	-	-	-	0.5	-	1
Emericella quinquixiani	ML 514	-	1	-	-	0.5	0.8	0.5	1.5
Emericella spectabilis	ML 488	0.5	1.5	0.5	1	0.5	0.8	-	1.5
Fusarium oxysporum	F 2.27	1	1.5	0.5	0.5	-	1.6	-	
Geotricum conidium	F 2.8	0.5	1.6	-	0.5	-	0.5	-	1.8
Penicillum corylophilum	ML 457	-	1	-	-	-	-	-	1.2
Penicillum corylophilum	ML 369	-	0.8	-	-	-	-	-	1
Penicillum corylophilum	ML 107	0.5	1.5	-	0.5	-	0.5	-	1
Penicillum decumbens	ML 109	0.5	1.2	-	-	0.5	0.8	0.5	1.4
Penicillum decumbens	ML 159	-	1.2	-	0.5	-	-	-	1
Penicillum decumbens	ML 155	0.5	1.2	-	0.5	-	-	-	1
Penicillum echinoulatum	ML 291	-	-	-	-	-	-	-	-
Penicillum italicum	ML 332	-	0.8	-	0.5	-	-	-	1
Penicillum italicum	ML 319	0.5	0.5	-	0.5	-	-	-	0.8
Penicillum minioletum	ML 172 A	0.5	1.3	-	0.5	-	0.5	-	2.1
Penicillum minioletum	ML 172 B	0.5	1.2	-	0.5	-	0.5	-	1.2
Penicillum polonicum	ML 329	0.5	2	0.5	0.5	0.5	1	0.5	1.5
Penicillum adametzioides	F 2.30	0.5	1	0.5	0.5	0.5	0.8	-	1.5
Penicillum brevicompactum	F 2.29	0.5	1.2	-	-	-	-	-	1.2
Penicillum brevicompactum	F 2.5	0.5	1.5	-	0.5	-	0.5	-	1.2
Penicillum echinolatum	F 1.5	-	1.5	0.5	-	0.5	-	-	2.2
Penicillum echinolatum	F 1.4	1.2	1.6	0.5	0.5	-	0.5	-	1.2
Penicillum olsonii	F 1.17	-	1.8	-	0.8	0.5	-	-	2
Penicillum olsonii	F 1.9	1	1.5	-	-	-	0.5	-	0.8
Penicillum sclerotiorum	F 2.26	0.5	1.8	-	1.5	1	1.5	0.8	2
Penicillum sclerotiorum	F 2.28	1	1	-	0.5	-	0.5	-	1.5
Rhizomucor	ML 296	0.5	1	-	0.5	-	-	-	1.3
Rhizomucor	ML 295	-	1	-	0.5	-	-	-	2
Rizophus stolonifer	F 2.18	-	1.2	-	0.5	1	0.5	-	1.5
Rizophus stolonifer	F 2.19	-	1.8	-	-	-	0.5	-	1
Rizophus stolonifer	F 2.23	-	0.5	-	0.5	0.8	0.5	-	1.5

The chemicals most relevant were the monoterpene hydrocarbons, ranging from 88.35% (EO L1) to 98.07% (EO AM). The oxygenated monoterpenes ranged between 11 and 1.9%. The oxygenated monoterpenes of lemon EOs were four/five times of those EO AM and EO MA, the less active among all the EOs tested in this study. Sesquiterpene hydrocarbons were detected in minimal percentages in lemon EOs (detected at 0.79% as maximum concentration), while only traces were detected in EO MA and none in EO AM. D-limonene resulted to be the major component (95-62%). α-Pinene, β-pinene and sabinene were quantitatively appreciable in lemon EOs, while traces were found in the others EOs. Among oxygenated monoterpenes, terpineol, citral and geraniol were in greater amounts in the EOs with higher antifungal activity.

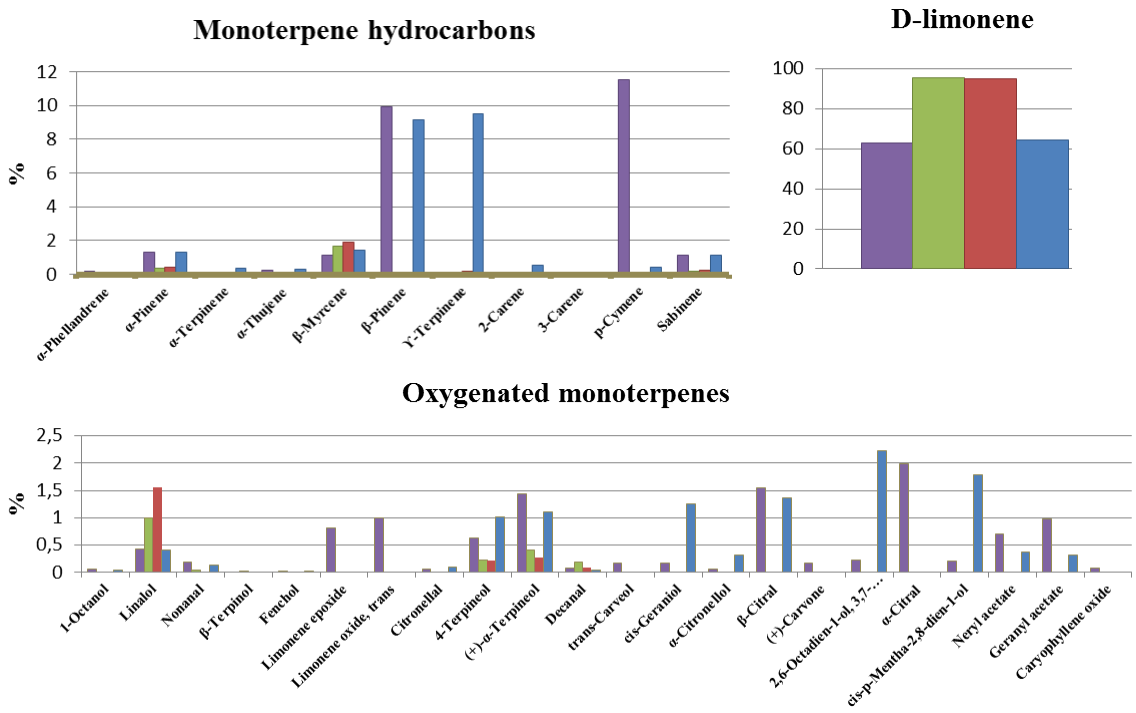


Figure 5.1. Quantitative differences of monoterpene hydrocarbons, D-limonene and oxygenated monoterpenes among EO L1 (purple), EO L2 (blue), EO AM (green) and EO MA (red). Values represent percentage of area of picks resulting from GC-MS analysis.

In conclusion, lemon EOs (EO L1 and EO L2) showed an in vitro antifungal activity clearly higher respect to both oranges and citrus hybrid EOs. The differences in the chemical composition might be imputable to the different antifungal activity.

In particular, the higher presence of oxygenated monoterpenes in the EOs with higher antifungal activity suggests that this group of compounds might be responsible of the fungal inhibition.

These results confirm previous studies showing antimicrobial activity due to oxygenated monoterpenes (Fisher *et al.*, 2008; Settanni *et al.*, 2014), besides monoterpene and sesquiterpene hydrocarbons could act sinergically in inhibiting microbial growth (Burt, 2004).

In conclusion, the higher antifungal activity was registered in presence of lemon peel EOs, which are indicated as an effective tool to control fungal decay in foods, even if further studies are necessary for their application in food matrices.

Concluding remarks

This chapter discusses the main findings of this research thesis in the context of the applications of essential oils as multi-target compounds for novel food safety strategies, such as active packaging technologies.

The main findings concern foodborne bacteria and molds inhibition by EOs, their chemical characterizations and application in packaging materials.

On a microbiological point of view, *S. aureus, L. monocytogenes, Salmonella enterica, Enterobacter* spp. and *E. coli* were all inhibited by *Citrus* EOs (lemon and mandarin EOs), even if to different extent. Moreover, the inhibitions registered were found to be *strain-dependent*.

In general, EOs were *more effective against Gram-positive than Gram-negative bacteria*. These findings might be related to the presence of the outer membrane in Gram-negative bacteria, which provides a strong impermeable barrier. Within the Gram-negative strains, *Salmonella* showed a lower sensitivity than *Enterobacter*.

The results achieved by fluorescence viability tests and PMA-qPCR indirectly confirmed the mode of action of EOs at the membrane level. The hydrophobicity of EOs affects the lipid bilayer of microbial cells, compromising the cell membrane, and resulting in a viable but not cultivable state of cells when sub-lethal doses are used.

A novel method based on *PMA-qPCR* was applied and resulted to be *able to discriminate between live and dead cells* after EO treatments. It does not affect live cells neither the efficiency of DNA amplification, even if the effectiveness of PMA in detecting only live cells may be saturated by increasing cell numbers.

The antimicrobial activity was assessed as well against foodborne moulds, showing a wider spectrum of inhibition of lemon EOs compared to both oranges and citrus hybrid EOs.

Regarding the chemical characterization of *Citrus* EOs all the compounds identified by GC-MS (42-36 varying among the different EOs) belonged to *monoterpene hydrocarbons*, *oxygenated monoterpenes* and *sesquiterpene hydrocarbons*.

In all cases, the monoterpene hydrocarbon fraction dominated all EOs, while sesquiterpene hydrocarbons constituted the quantitative percentage less relevant. *D-limonene* was the compound at the higher concentration, characterizing *Citrus* EOs.

Qualitative and quantitative differences in the EOs composition have been reported among different *harvest stages* of lemon fruits and among experimental and commercially *extracted samples*, in case of mandarin. These factors influence the chemical composition and then deeply affect the antimicrobial performance of EOs.

The comparison of the chemical composition of EOs suggested that the compounds mainly implicated in the process of bacterial inhibition could be the *oxygenated monoterpenes*, since they have been detected in effective EOs in concentrations four/five folds higher than ineffective EOs.

Furthermore, the antibacterial effect of the EOs was maintained when they were incorporated into active *films* of interest in food packaging. This behavior was confirmed for *chitosan* (CH) based films.

The highest significant antibacterial effect evidenced in case of the *incubation at 8°C* may be related to the influence of the temperature in promoting the permeability of cell membranes and, thus, dissolving more easily EOs in the lipid bilayer when low

temperatures occur. This consideration opens new good perspectives for refrigerated shelf-life applications.

Considering further food-applications, it seems that the greatest limitation to the extensive use of EOs and their components will be the interactions with food components and the strong flavor and aroma.

In this way, inactivation of EOs by lipids and proteins could be reduced by technological means, such as packaging material by which a controlled release can be obtained. Thus, the use of essential oils for packaging technologies may be promising for certain niches in the food industry to prevent growth of spoilage organisms or to reduce viable numbers of pathogens.

In this way, the antibacterial and antifungal properties of EOs, together with the chemico-physical properties of the material constituting the packaging material and acting as a selective barrier to gas transport, can be the goal of a *hurdle technology* applied to food to extend its food safety and commercial shelf-life.

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Scientific production during Ph.D.

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Acknowledgements

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe is as good as dead: his eyes are closed."
(A. Einstein)

Beside this citation, I see the example of my father, ***Rosario***, who raised on me the desire of experiencing and knowing. Thanks to this stimulating and positive perspective he passed on me, I crossed over all the challenges of my life, including the ones of my Ph.D.
Therefore, my first, deep, sincere and full of love though goes to him.

No one of the results of this thesis would have been possible without the support and the help received from several persons.
Firstly, I am deeply grateful to all of the Microbiology Unit of the Dipartimento di Scienze Agrarie e Forestali dell'Università di Palermo, especially to my supervisor, Prof. ***Giancarlo Moschetti***, and to my co-tutor Dr. ***Nicola Francesca.*** A special thank goes to Prof. ***Luca Settanni*** that supported me since my degree thesis. I am grateful to my colleagues ***Antonio Alfonzo, Ciro Sannino, Raimondo Gaglio, Rosa Guarcello, Alessandra Martorana, Marcella Barbera, Margherita Cruciata, Milko Sinacori, Anna Nasca, Giusi Ventimigia, Selene Giambra*** and ***Gaetano Conigliaro***, with whom I daily shared happiness, sorrow and many coffees during these years. All of them have helped me at different extent, making my work at lab always pleasant and fruitful.
I want to thank as well all the research groups I collaborated with during my Ph.D. Firstly, all the ones of my department, Dipartimento di Scienze Agrarie e Forestali dell'Università di Palermo, in particular Prof. ***Maria Antonietta Germanà***, Prof. ***Eristanna Palazzolo***, Dr. ***Onofrio Corona***, Dr. ***Michele Panno***, Prof. ***Franca Barone*** and Dr. ***Vittorio Farina***.
I am deeply grateful to Dr. ***Valeria Guarrasi*** (Istituto di Biofisica – Consiglio Nazionale della Ricerca of Palermo) for helping me in exceeding of research difficulties. I thank ***Marta Moschetti*** for the analyses she did and Prof. ***Pier Luigi San Biagio***.
I thank Prof. ***Caterina Mammina*** and Dr. ***Aurora Aleo*** for their warm welcome in their lab at Dipartimento di Scienze per la Promozione della Salute e Materno Infantile "G.D'Alessandro" (Palermo) during some experimental steps.

I want to thank Prof. ***Ana Jiménez Belenguer*** of the Departmento de Biotecnologia, Área Microbiologia, E.T.S.I.A.M., Universitat Politècnica de València, for supporting me throughout my first staying in Spain and for the stimulating discussions we had. I would like to thank Prof. ***Maria Vargas*** and ***Angela Perdones*** (Instituto Universitario de Ingeniería de Alimentos para el Desarrollo, Departamento de Tecnología de Alimentos, Universitat Politècnica de València) for the prolific collaborations concerning food coatings.

I am deeply grateful to Dr. ***Gloria Sanchez Moragas*** (Biotechnology Department, IATA-CSIC, Spain) for her kindness, for teaching me about novel viral and bacterial technics, for supporting me throughout my last staying in Spain, always showing patience and positive point of view. Thanks to Prof. ***Rosa Aznar Novella*** for welcoming me in her laboratory.

A special thank goes to all the ones of the Instituto Agroforestal Mediterráneo, Univeritat Politecnica de Valencia. Thanks to ***Manolo, Carlos, Carmina, Amparo*** that supported me during my staying at Valencia on both personal and scientific point of view. Thanks to ***Mercedes*** for her kindness, her advices and fruitful collaborations concerning essential oils.

Finally, I want to thank my collegues at IATA-CSIC, ***Alba, Javier, Sara, Anto, Fani, Jiri, Lucia, Aurora, Ceci, Adri, Irene*** and ***Carol*** for sharing protocols, making pleasant my staying and for your willingness to nights out.

A special thanks to all of my friends of Palermo for supporting me during these years: ***Ivan, Pinus, Ana, Andrea, Eliana, Giulia, Oscar, Andrea, Giulia, Mario, Laura, Mariella, Dana, Marco, Vivi, Carlo, Chicca***. A special deep thought goes to ***Natalia*** that always give to me positive vibrations.

Finally, a gratefully hug to my lovely family, firstly to my mom, ***Marinita***, my brother, ***Dario***, my grand-mother, ***Raimonda,*** and my father-in-law, ***Rosario***, for caring me all the time and for supporting me in that professional experience even when I was abroad.

I wish you all to always experience the mysterious,
to pause to wonder and to stand rapt in awe!!!

A huge thank to all of you that make this experience such outstanding!!!